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Metabolic Engineering and Transhydrogenase Effects on NADPH Availability in *Escherichia coli*

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ABSTRACT

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The ultimate goal in the field of metabolic engineering is improving cellular processes in a rational manner using engineering design principles and molecular biology techniques. The syntheses of several industrially useful compounds are cofactor-dependent. The reducing equivalent NADPH is required in several enzymatic reactions leading up to the synthesis of high-value compounds like polymers, chiral alcohols, and antibiotics. However, it's a highly costly compound with limited intracellular availability. This study focuses on the genetic manipulation of a whole-cell system using the two transhydrogenase isoforms *pntAB* and *udhA*. Two model systems are used: 1) the production of (S)-2-chloropropionate and 2) the production of poly(3-hydroxybutyrate). Results suggest that the presence of *udhA* increases product yield and NADPH availability while the presence of *pntAB* has the opposite effect. A maximum product yield of 1.4 mole-product/mole-glucose was achieved aerobically in a *pntAB*-deletion strain with *udhA* overexpression, a 150% improvement over the wild-type control strain.

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Chapter 1

1. Introduction

1.1 Metabolic Engineering

1.1.1 Introduction to metabolic engineering

Metabolic engineering is a highly multidisciplinary field, combining the likes of chemical engineers, bioengineers, molecular biologists, computational scientists, biochemists, and analytical chemists, among others (Nielsen 1997, Yang 1998, Cameron 1993). The ultimate goal of the field is to improve upon cellular processes in a controlled and deliberate manner through the application of engineering design principles and molecular biology techniques. Desired effects are commonly achieved via pathway manipulation and genetic modifications.

Over the years, advances in recombinant DNA technology have allowed scientists and engineers to circumvent dependency upon random mutagenesis and instead allow for highly rational and precise approaches to genetic modification and subsequent screening processes (Koffas 1999). The inception of this move from random mutagenesis and selective screening to a highly systematic approach of pathway manipulation was first introduced in the 1990s by Bailey (1991) and Stephanopoulos and Vanillo (1991). As the field has advanced, so has the ability of scientists and engineers to incorporate rigorous and well-defined mutations, thereby creating strains with definitive genetic backgrounds. Recombinant DNA techniques, in this effect, were one of the tools that facilitated the onset of directed cellular modifications, alongside the emergence of extensive databases

on gene expression, genomic maps, and enzyme structure and refined analytical techniques (Keasling, 2010).

This rational approach to pathway manipulation is the basis of metabolic engineering and has been defined by various authors as "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (Bailey, 1991), "the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology" (Stephanopoulos, 1998), and "the purposeful modification of intermediary metabolism using recombinant DNA techniques" (Cameron, 1993), to name a few.

Clearly, recombinant DNA technology plays a large role in the execution and advancement of the field, though it is by all means not the sole factor. Like other engineering disciplines, metabolic engineering entails two defining mechanisms: synthesis and analysis (Koffas 1999, Stephanopoulos 1998). As far as synthesis is concerned, scientists and engineers must rely upon molecular biology techniques to alter enzymatic, transport, and regulatory cellular functions by introducing new gene expression cassettes, modifying or deleting existing genes, and/or amplifying endogenous enzymes.

In order to arrive at a rational basis of identifying targets for manipulation, researchers must have an understanding of the physiological workings of the species in question, as the metabolic activities of cells are a highly intricate and networked web of approximately 1,000 enzyme-catalyzed reactions and selective membrane transport systems (Bailey, 1999). Metabolic control analysis and biochemical systems theory are

two examples of available methods for identifying bottlenecks in pathways upon which targets for manipulation are identified. Both are mathematical methods for modeling pathways and systems. They provide insight on the effects of enzymatic levels on fluxes through pathways and metabolite concentrations (Keasling, 1999).

It is necessary to practice caution when planning pathway manipulation, as changes can place a great amount of metabolic burden on cells, and pathways critical to cell survival must remain intact. Consequently, alterations must be planned out and chosen in a systematic and deliberate manner. Pathway analysis and analytical techniques such as metabolic flux analysis are employed, and a reiterative process wherein genetic changes are made, downstream effects are analyzed, and further genetic changes are decided upon, is used to refine the mutant strain at hand (Bailey, 1999).

1.1.2 Metabolic engineering tools

As mentioned previously, advancements in the field of metabolic engineering are dependent upon molecular biology techniques such as recombinant DNA, genomic maps, enzymatic databases, and analytical techniques, among others (Keasling, 2010). True to its multidisciplinary foundation, the field calls for several experimental, computational, and analytical tools.

1.1.2.1 Gene expression tools

The development of refined recombinant DNA techniques spearheaded metabolic engineering's turn from random mutagenesis and screening to that of rational and directed genetic modifications. Molecular biology techniques are widely used. Among

those frequently used are cellular transformation systems, plasmids, transcriptional control methods, and 'tunable' promoters (Stafford, 2001). Keasling (1999) identified four critical characteristics that define ideal gene expression tools. The distinguishing features are as follows: 1) promoters that exhibit established and consistent control of cells with a linear response to added inducers if they are inducible promoters, 2) the ability to regulate multiple gene expressions at varying levels in a simultaneous manner, 3) gene-carrying vectors that perpetuate in the host indefinitely or for several generations with minimal selective pressure, and 4) minimal metabolic burden on the host.

Cloning vectors are used to shuttle foreign DNA encoding desired enzymes associated with inherent or new metabolic pathways into host cells. Genes that encode the enzyme are commonly put under the control of inducible promoters on high-copy plasmids. These plasmids are introduced into host cells via cloning vectors, where they replicate, perpetuating the foreign DNA. Plasmids are categorized according to incompatibility groups. Those in the same incompatibility groups are incapable of coexisting stably in the same cell. Plasmids are also characterized by their copy number, which is an indicator of the number of plasmids that can be maintained within the cell. High copy number plasmids have copy numbers of approximately 100 per cell while low copy number plasmids have 1-5 per cell (Keasling, 1999).

Promoters are used to exercise control over gene expression. Native promoters of heterologous genes are more often than not replaced with a host-specific promoter. A linear response to inducer concentration is ideal wherein little to no gene expression is observed in the absence of the inducer and a direct, linear correlation is observed in the presence of the inducer. Inducible promoters are of great utility as they allow for

inducible expression of desired pathways. A commonly used expression system is the *lac* expression system. A variety of plasmids containing derivatives of the lactose-inducible promoter are now widely available. Promoters that are induced by starvation are of particular utility when applied to bioremediation of environmental contaminants.

Oftentimes more than one enzyme controls the metabolic flux through a pathway (Stephanopoulos, 1997). In such cases, directed modifications are precluded by the need to express more than one gene in the given pathway. Coordinated expression of multiple genes can be achieved effectively using multiple inducible promoters, each controlling a particular gene. A caveat of this approach is the need to add several inducers to the medium. This drawback can be circumvented by implementing a method developed by Jensen and Hammer, which places genes under the control of promoters of different strengths. However, this approach can be more labor intensive when it comes to the construction of promoters (Keasling, 1999).

1.1.2.2 Analytical tools

Common analytical tools for detecting metabolite concentrations are high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) for detecting metabolite concentrations.

1.1.2.3 Mathematical tools

Computational and mathematical tools are essential to rational pathway modulation. When coupled with experimental procedures, they facilitate pathway flux and flux distribution analysis. Modeling of pathways in this manner allows for metabolic

flux analysis, wherein metabolic flux is defined as “the rate at which substance flows from the initial substrate to final pathway product” (Byrne, 2009). An example of this is the rate of conversion from glucose to pyruvate during glycolysis. Flux is a fundamental aspect of metabolic pathways and cellular physiology, and its control is necessary in order to maintain metabolite concentrations that remain with appropriate ranges for cellular survival (Byrne 2009, Stafford 2001). Current methodologies for determining metabolic flux are based upon information such as intracellular/extracellular metabolites and the rate of substrate uptake, among others, for stoichiometric balances (Stafford, 2001).

Metabolic control analysis is used to determine the importance of enzymes in metabolic flux regulation. This mathematical model expresses the sensitivity of metabolic flux to changes in enzymatic activity levels and is described in detail by Stephanopoulos et al. (1998). In essence, it examines the relative importance and effect of enzymes on fluxes and metabolite concentrations. It is a powerful quantitative tool when examining steady state properties of biochemical reactions both theoretically and experimentally.

1.1.3 Metabolic engineering applications

The primary objective of metabolic engineering is efficient and robust production of desired products for commercial uses. As such, the scope of the field is immense and relevant in pharmaceutical, environmental, chemical, food, and health care industries, to name a few, and can be grouped into the following five broad categories, as delineated by Cameron et al. (1993).

1.1.3.1 Enhanced yield of native products

In this instance, metabolic engineering is applied with the intent of increasing the productivity of naturally produced compounds of host microorganisms. Native compounds such as catabolic end products, antibiotics, vitamins, amino acids, and polymers, among others, hold great value in industrial realms (Cameron, 1993). Enhanced productivity is a major incentive as it allows for cost-effective commercial applications. Efficient ethanol production, for example, has been studied extensively by several groups (Brau 1987, Tolan 1987, Ingram 1987). Metabolic engineering of antibiotics (Chen 1988, Hutchinson 1992) is particularly attractive from a technical point of view as they are more often than not positively regulated and have a clustered arrangement of antibiotic genes, allowing for the overexpression of antibiotic gene regulators and more effortless cloning when designing genetic modifications to host organisms (Chater, 1990).

1.1.3.2 Extended substrate range for host utilization

Metabolic engineering has also been used to extend the substrate range for host organism growth and product formation. By diversifying substrate options, organisms can be cultured on more cost-efficient substrates, like in the case of ethanol production on cellobiose and cellotriose (Wood, 1992) or biosurfactant production on industrial wastes such as whey and lactose (Koch 1988, Kumar 1992).

1.1.3.3 Enhanced degradation of toxic products

Another beneficial application of metabolic engineering is the introduction of novel catabolic processes for toxic chemical degradation. Modifications such as these are difficult, as they can be associated with unfavorable energetics of substrate utilization in addition to challenging substrate transport phenomena, substrate toxicity, and low substrate concentration (Tong, 1993). Engineering microbes for bioremediation is an attractive means for the removal of industrial pollutants as it provides an alternative, green, and economical means of treatment. Examples include bioadsorbents for sequestering heavy metals (Sousa, 1998) and the catabolism of camphor and naphthalene (Chakrabarty, 1974).

1.1.3.4 Enhanced yield of nonnative products

In some situations the inactivation or overexpression of unique enzymes or pathways activates alternative enzymes or pathways capable of restoring order and ultimately negating the desired effect. This notion is especially apparent in the production of primary metabolites in the highly regulated and controlled central metabolism (Yang, 2007). Fortunately, host organisms lacking the necessary metabolic pathway are still capable of producing target metabolites. On such occasions, the necessary pathway is introduced to the non-native producing host cells in order to arrive at the desired product in an efficient manner. A large amount of work in this area of metabolic engineering has been in the realm of antibiotics with a primary focus on the discovery of novel, hybrid antibiotics (Cameron 1993, Hopwood 1985). Another example is the production of polymers. An example is polyhydroxybutyrate (PHB) in

Alcaligenes eutrophus, which has been cloned and expressed in *Escherichia coli* (Sanchez, 2006).

1.1.3.5 Enhanced cellular properties

The objective with this application of metabolic engineering is enhancing cellular fitness and hardiness by improving host properties ranging from increased growth rate to toxic metabolic intermediate resistance, optimized substrate utilization, and ultimately enhanced productivity and product yields. Examples include growth in an oxygen-limiting environment (Bailey 1990, Khosla 1988) and insulin response to glucose (Hughes, 1992).

1.1.4 Recent advances and applications in metabolic engineering

In this day and age metabolic engineering is a valuable tool that is utilized across several sectors in industry. The development of increasingly refined and intuitive engineering tools has fueled the growth of the field itself, and note-worthy advances are being made when it comes to translating the discipline into tangible products and processes of high value and practical use. Two applications that have generated considerable interest as of late reside in the pharmaceutical and energy industries. This section focuses on recounting the impact of the field in these two industries, among others.

1.1.4.1 Natural product drug discovery

Khosla and Keasling published a recent review article highlighting the development of metabolic engineering along with its applications to drug discovery and development in 2003. The utility of metabolic engineering techniques in the pharmaceutical industry is immense. The turnaround rate in the drug discovery pipeline is lacking. For every successful candidate that makes its way to market, there are, on average, five to ten thousand failed compounds behind it (Khosla, 2003). Throw in the fact that it takes about 10-15 years for a successful drug to go from ground zero to market on top of that, and one can see the need for a rational means of producing viable drug candidates from cost-efficient starting materials in large quantities and in heterologous hosts (Khosla, 2003).

The majority of effective drugs are of natural origin or a derivative of natural products. In fact, in the 1990's approximately 80% of drugs were either natural or naturally-derived products (Li, 2009). Examples of such are potent anticancer (doxorubicin, taxol), antibacterial (erythromycin, streptomycin), and immunosuppressant (cyclosporine) drugs. However, the time frame spanning from 2001 to 2008 experienced a 30% drop in natural product-based drug design (Li, 2009). What spurred this noticeable trend? A prominent challenge the pharmaceutical industry faces is related to technical shortcomings when synthesizing and identifying new compounds with desirable qualities. Fortunately, this decline is not projected to be long lived, with new technologies such as metabolic engineering, synthetic biology, and smart screening methods for new natural product drug discovery on the horizon.

The use of heterologous hosts for new drug production is dependent upon the introduction of new genes or manipulation of inherent genes in biological pathways. It entails rational genetic overexpression, underexpression, or deletion in metabolic pathways in order to streamline product synthesis and avoid bottlenecks within inherent and/or newly introduced pathways. The past decade has been marked by metabolic engineering-fueled discoveries in the pharmaceutical industry (Khosla, 2003). Common classes of natural products, as delineated by Khosla et al., wherein metabolic engineering has made an impact are presented below.

1.1.4.1.1 Isoprenoids

Isoprenoids are a diverse class of natural compounds composed of over 50,000 known compounds including terpenoids and carotenoids (McCaskill, 1997). Carotenoids are powerful antioxidants, and research has shown that they are effective in preventing cardiovascular disease and cancer (Sandmann, 1999). They are naturally-occuring compounds synthesized by plants and microorganisms. Sandmann et al. overcame their limited availability via heterologous expression of carotenoid genes from various host species in *Escherichia* coli.

Terpenoids are effective as antifungal, antibacterial, and anticancer agents (Khosla, 2003). Microbial systems such as *Escherichia coli* and *Saccharomyces cerevisiae* are characterized by poor expression of the terpene cyclase genes (Martin, 2001). However, these limitations can be overcome when applying metabolic engineering principles. The anti-malaria drug, artemesinin, was the subject of a study by Martin et al. in a recent paper published in 2003. Martin et al. engineered the mevalonate

pathway in *Escherichia coli* by synthesizing and expressing the gene encoding amorphadiene synthase to increase production of the artemisinin precursor amorphadiene and achieved 24 μg equivalent/mL.

1.1.4.1.2 Polyketides

Polyketides are a class of natural metabolites derived from acyl-CoA monomers. Commonly used polyketides range from antibacterials (erythromycin, tetracyclin) to anticancer agents (doxirubin) to cholesterol-lowering agents (lovastatin) and agrochemicals (spinosyn) (Khosla, 2003). Their synthesis is controlled by a polyketide synthases. The greatest limitation when examining and manipulating the synthesis of polyketides are due to challenges associated with their natural host. Directed engineering approaches are being used to for high productivity synthesis of these compounds. An example of such is the transfer of biosynthetic capabilities into a heterologous host. The horizontal transfer of polyketide pathways into heterologous hosts has been examined by Pfieifer et al. (2001), particularly in the microbial hosts *Streptomyces coelicolor* and *Escherichia coli*. The overproduction of known metabolites such as the anti-tumor agent epothilone in *Streptomyces coelicolor* has also been documented (Khosla, 2003).

1.1.4.1.3 Non-ribosomal peptides

Metabolic engineering principles have been applied to the production of non-ribosomal peptides, perhaps the most well-known of which is penicillin. An example in this area is the development of a direct fermentation process for the production of industrially useful intermediates (Khosla, 2003).

1.1.4.1.4 Carbohydrates

In addition to fueling advances in the production of the aforementioned compounds, metabolic engineering has made strides in the synthesis of vitamins like folate and riboflavin as well as sugars such as mannitol and sorbitol, among others. The focus in this area is the discovery of glycosidic drug candidates. The biosynthesis of these compounds revolves around redox, amino transfer, and methyl transfer processes (Khosla, 2003).

1.1.4.2 Biofuels from renewable resources

Due to rising energy costs and environmental concerns such as pollution and climate change, there has been a push in engineering biofuels from renewable sources. Renewable resources like agricultural crops, biomass, and waste materials are used to generate biofuels (Cheng, 2011). Currently, the most commonly used biofuels are those derived from starches (corn) or sugar cane, while the most widely used biodiesels are those from vegetable oils or animal fat (Fortman 2008, Cheng 2011). The conversion of renewable resources, like biomass-derived sugars, into transportation fuel was first conceptualized back in the 1970s (Fortman 2008, Stephanopoulos 2007). Over the years, great strides have been made in the field, alongside advancements in genomics and metabolic engineering.

Bioethanol is the chief biofuel of the worldwide market, used to make gasoline-ethanol mixtures like E85, consisting of 85% ethanol and 15% gasoline. In 2008, bioethanol production worldwide stood at 17.34 billion gallons (Cheng, 2011). As mentioned previously, it is commonly produced from sugar cane or corn feedstocks.

Unfortunately, sources like these place considerable strain on limited agricultural resources. Furthermore, bioethanol is characterized by low energy density, containing 30% less energy content than gasoline (Atsumi 2008, Keasling 2008). Barring such hindrances, an intuitive approach to bioethanol production utilizes glycerol, a common biodiesel waste, in the production process. Glycerol contains highly reduced carbons that are capable of being converted into fuels or other reduced products such as 1,2-propanediol (Clomburg and Gonzalez, 2011) at greater yields than those associated with common sugars (Gonzalez, 2008). *Enterobactor aerogenes* HU101 has shown promising results utilizing glycerol for ethanol production (Ito, 2005) and Gonzalez et al. (2008) have characterized key pathways for the fermentative metabolism of glycerol in *E. coli*, indicating potential for producing biofuel from biodiesel waste and presenting it as a platform for the conversion of glycerol into biofuels.

More sustainable alternatives are biofuels derived from lignocellulosic materials; the most readily available and renewable organic resource at our disposal (Aristidou, 2000). Lignocellulosic materials present several advantages. For one, its production is not reliant upon food-based agricultural resources such as corn or sugarcane. It is also a readily available biopolymer, and it can be engineered to produce fossil-fuel replacements via new biosynthetic pathways (Keasling, 2008). These materials can be used for biofuel production because they have high cellulose and hemicellulose content. Cellulose is a high molecular weight and linear glucose polysaccharide containing over 10,000 sugar units, while hemicellulose is a low molecular weight and heterogeneous polysaccharide containing 100-200 sugar units. Hemicellulose monomers include hexoses such as glucose, galactose, and mannose, as well as pentose such as xylose and

arabinose. Studies are being done on the conversion of these source materials into economically viable biofuels.

Isopropanol, a secondary alcohol produced by microbes (Osburn, 1937), has been explored as a sustainable biofuel. It is naturally produced in several species of *Clostridium* and has been studied extensively in these species. However, due to limited knowledge in regards to metabolic regulation in *Clostridium* strains as well as laborious genetic manipulations, a more optimal approach entails moving and engineering a synthetic pathway into a more desirable host. In *Clostridium* species isopropanol is produced from the acetone pathway, and a previous study by Bermejo et al. (1998) moved four genes from *Clostridium acetobutylicum* into *Escherichia coli* and resulted in a mutant strain capable of producing near-equivalent levels of acetone as the native host. Hanai et al. engineered a synthetic pathway in *E. coli* by introducing genes from *Clostridium* species into *E. coli* K-12 MG1655 to produce isopropanol from acetyl-CoA via acetone. The resultant strain produced a high titer of isopropanol (81.6 mM), which exceeded that of the best reported strain, *Clostridium beijerinckii* (30 mM).

n-Butanol is another biofuel under investigation. Its energy content (27 MJ/L) is similar to that of gasoline (32 MJ/L) (Atsumi, 2008). Its native host is *Clostridium acetobutylicum*. A previous study by Fontaine et al. examined n-butanol production in by cloning and expressing the recombinant AdhE2 gene, responsible for n-butanol production, into *E. coli*. Another metabolic engineering approach entails deleting competing host pathways; for example, pathways that compete with n-butanol for Acetyl-CoA and NADH. In fact, n-butanol production has been shown to increase three-fold

over wild-type levels when *ldhA, adhE, frdBC, pta,* and *fnr* are deleted from wild-type *E. coli* strains (Atsumi, 2008).

These are just a few examples of strides being made in biofuel production from renewable resources. Metabolic engineering provides valuable tools for engineering heterologous hosts for the cost effective and efficient production of biofuels, providing sustainable alternatives over the traditionally and widely-used ethanol and gasoline. When examining advances in this field, focus is placed on cost-effective approaches that alleviate environmental burdens, and in order to achieve this goal, product yield, titer, and productivity must all be improved.

The field can be further extended by considering waste and byproducts in the biofuel industry, an example of which is glycerol. Crude glycerol accumulation is observed during the production of biodiesel and bioethanol and in such large quantities that disposal costs become a major factor. While accumulated glycerol can be converted into higher value products via biological or chemical transformations, its purification process is costly and uneconomical. However, *E. coli* has been shown to metabolize glycerol fermentatively. As such, engineering *E. coli* for the production of chemicals and fuels shows promise. (Yazdani, 2010)

1.1.5 Cofactor and pathway engineering

A commonly implemented approach in metabolic engineering begins by first pin-pointing the rate-limiting step in a given pathway and then manipulating enzyme levels to moderate production. While this marks a traditional approach in the field, it is not without its limitations. The method takes into account enzymatically controlled rate-

determining steps in pathways. However, once the bottleneck is alleviated through enzyme overexpression, deletion, or addition, further considerations may need to be taken into account. A lack of a complete understanding of central cellular metabolism and the integrated nature of its numerous processes is a common obstacle in attempts to design optimal pathways for efficient production.

Cofactors, for instance, are known to play a hand in numerous biochemical reactions. Cofactor engineering presents itself as a powerful tool in the field of metabolic engineering. When examining cofactor-dependent systems, a logical approach would incorporate cofactor manipulation.

Cofactors are non-protein components that provide enzymes with chemically versatile functions. They are capable of binding to enzymes in a reversible manner without undergoing any major chemical alterations in the process (Garrett, 2010). Therefore, unlike metabolites, cofactors are able to undergo a change in state during an enzymatic reaction while maintaining the capability of returning to their original state via a regenerative reaction. However, the high cost associated with such cofactors is a prominent roadblock in commercial applications (Zhao, 2003). Thus, efficient and cost-effective cofactor regeneration is needed.

Cofactor pairs commonly act as donors and/or acceptors of reducing equivalents *in vivo*, effectively shuttling electrons and undergoing oxidation and/or reduction. Common cofactor pairs are NADH/NAD+, CoA/Acetyl-CoA, NADPH/NADP+, and ADP/ATP, among others. These pairs undergo reversible transformations between their oxidized and reduced states, and the ratio between the pairs is known to affect pathways.

As a result, they can be manipulated and used as a control parameter when altering and modulating cellular metabolism.

1.1.5.1 NADH/NAD+ cofactor pair

Previous work has been performed on investigating the effects of cofactor engineering. The cofactor pair NADH/NAD+ has a major role in microbial catabolism wherein the nicotinamide adenine dinucleotide cofactor (NAD+) oxidizes a carbon source and produces the reducing equivalent NADH. NAD+ regeneration is essential to cellular survival. NADH/NAD+ cofactor engineering has been effective in boosting the productivity of industrially-relevant compounds such as ethanol and 1-2, propanediol (Berrios-Rivera, 2003). This laboratory, for example, has examined the role and effects of NAD+, known to take part in over 300 redox reactions (Berrios-Rivera, 2002). 1,2-propanediol production was achieved by overexpressing the methyl-glyoxal synthase enzyme from *Clostridium acetobutylicum* and the glycerol dehydrogenase enzyme from *E. coli*. A lactate dehydrogenase deletion was also incorporated to eliminate a competing NADH-dependent pathway. This study found that by increasing NADH availability under anaerobic conditions, metabolic flux redistribution followed and resulted in a higher ethanol to acetate ratio (Berrios-Rivera, 2003).

1.1.5.2 CoA/Acetyl-CoA cofactor pair

The cofactor pair CoA/Acetyl-CoA has also been under investigation. Both CoA and Acetyl-CoA are involved in several metabolic pathways, acting as essential intermediates and regulators (Chohnan, 1997). For instance, acetyl-CoA acts as a

substrate during the synthesis of esters (Horton, 2003) and also plays a role in the production of poly(3-hydroxybutyrate), PHB, a biodegradable polymer (Lee 1995, Sanchez 2006, Wong 2008). Vadali et al. performed a study on CoA and acetyl-CoA levels in an aerobic batch reactor along with the effects of overexpressing pantothenate kinase, an upstream enzyme that catalyzes the rate-limiting step in the synthesis of CoA (Song, 1992). They observed a 10-fold increase in intracellular CoA levels and a 5-fold increase in acetyl-CoA levels, resulting in carbon flux increase to acetate production pathway.

1.1.5.3 ADP/ATP cofactor pair

ATP is a common phosphorylating agent for supplying energy in several biochemical processes. The regeneration of ATP from ADP has been previously explored using both enzymatic (Chenault, 1987) and whole-cell (Endo 2001, Fujio 1985) based methods. Enzymatic methods are typically quite costly, as they call for costly substrates and enzymes. Fujio et al. performed studies on whole-cell ATP regeneration in *Brevibacterium ammoniagenes* cells grown in medium supplemented with magnesium ions and phytic acid, resulting in increased ATP formation.

1.1.6 Research objectives

Primary research encompasses metabolic engineering of pathways in *E. coli* to increase NADPH availability, allowing for higher productivity and product yields of NADPH-dependent and industrially relevant compounds. Therefore, the ultimate objective of this study is the application of rational genetic modifications using pathway

and cofactor engineering to characterize NADPH availability by determining product yield of cofactor-dependent products in *E. coli*. This objective is achieved using classic metabolic engineering strategies such as overexpression and deletion of specific enzymes.

In the course of this study, cofactor constraints are taken into consideration in efforts to attain high product yields and productivities when catalyzing the conversion of substrate to product. The cofactor pair of interest is NADPH/NADP+. The reducing agent, NADPH, is an important coenzyme required in several biological reactions. The implementation of whole-cell fermentation systems in this regard allows for more robust and cost-effective synthesis of compounds.

The effects of two transhydrogenases, *pntAB* and *udhA*, on NADPH availability are examined. *PntAB*, a membrane-bound, proton-translocating transhydrogenase, and *UdhA*, a soluble, energy-independent transhydrogenase, function in NADPH metabolism of *E. coli*. These two transhydrogenease isoforms transfer electrons between NAD and NADP.

Transhydrogenase mutants and overexpression plasmids are used to manipulate expression levels of these transhydrogenases. By modifying transhydrogenase expression levels, subsequent effects on NADPH availability are assessed in two unique model systems: 1) using the production of (S)-2-chloropropionate catalyzed by the NADPHdependent 2-haloacrylate reductase (CAA43), and 2) using the production of poly(3-hydroxybutyrate) (PHB), a biodegradable polymer.

Chapter 2

2. Materials and Methods

This chapter contains the general methods and protocols used to conduct the research presented herein. Material and methods are described in detail in corresponding sections.

2.1 Bacterial Strains and Plasmids

2.1.1 Strains and plasmids

The *E. coli* strains and plasmids used in this work are presented in Table 2-1.

2.1.2 Bacterial strain construction

Single *udhA* and *pntAB* transhydrogenase mutants and a double transhydrogenase mutant were constructed in wild type MG1655 *E. coli*. The *udhA* mutant was constructed using the one-step inactivation method first described by Datsenko and Wanner (2000). The primers used for introducing the kanamycin cassette in the *udhA* gene were as follows:

Forward: ATGCCACATTCTACGATTACGATGCCATAGTAATAGGGTGTA-GGCTGGAGC-TGCTTC

Reverse: GTTTCTAGCCCAATGTTCTGTAACGCCAGCGAATCGCATATG-AATATCCTCCTTAG

Table 2-1. List of *E. coli* strains and plasmids used in this study.

	Genotype	References
Strain		
MG1655	Wild type *E. coli* ($F^- \lambda^-$)	ATCC 47076
MBS601	MG1655 Δ*udhA*	This study
MBS602	MG1655 Δ*pntAB*	This study
MBS603	MG1655 Δ*pntAB* Δ*udhA*	This study
Plasmids		
pDHC29	Cloning vector, Cm^R	(Phillips et al. 2000)
pCAA43	2-Haloacrylate reductase gene from *Burkholderia sp*. in pTrc99A, Amp^R	(Kurata et al. 2005)
pAeT29	Encodes β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHB synthase enzymes from *Alcaligenes eutrophus,* Amp^R	ATCC 75207
p*PNTAB*	Proton-translocating pyridine nucleotide transhydrogenase gene *(pntAB)* from *E. coli*, in pDHC29, Cm^R	This study
pUDHAC	Soluble pyridine nucleotide transhydrogenase gene (*udhA*) from *E. coli* in pDHC29, Cm^R	This study

The underlined portion of the sequence is homologous to *udhA*, and the non-underlined portion is homologous to the kanamycin region of pKD4. This construct was denominated MBS601 and was constructed by Dr. Irene Martínez Basterrechea (Dr. San's lab, Rice University). The *pntAB* mutant was constructed using P1-phage transduction with the strain JW1594 from the Keio Collection as the donor strain (Baba, 2006). This construct was denominated MBS602 and was constructed by Dr. Irene Martinez Basterrechea (Dr. San's lab, Rice University).

The *udhA* overexpression plasmid, pUDHAC (Cm^R) was constructed by moving *udhA* from the overexpression plasmid, p*UDHA*K (Km^R) previously constructed by Sanchez et al. (2006). The *udhA* gene (1.6 kb) was digested with ApaI and SpeI and cloned into pDHC29(Phillips, 2000) multiple cloning site. This construct was constructed by Dr. Yipeng Wang (Dr. Bennett's lab, Rice University).

The *pntAB* overexpression plasmid was constructed by amplifying the *pntA* and *pntB* genes from *E. coli* MG1655 chromosomal DNA with 300 bp before and 20 bp after the sequence. The native promoter was included in the construct. The primers used for the construct were as follows:

Forward: TTATCTGCAGAAGTAGTGATTCGTGC

Reverse: ATTATATCGATCTCAGCAGAGGCCGTC

The forward and reverse primers included a PstI and ClaI restriction site (underlined), respectively. The PCR product was digested with PstI and ClaI and cloned into pDHC29 (Cm^R). This construct was constructed by Dr. Irene Martinez Basterrechea (Dr. San's lab, Rice University).

The 2-Haloacrylate reductase expression plasmid was constructed by amplifying the CAA43 gene from pET101-TOPO generously given to us by Kurata et al. The primers used for the construct were as follows:

Forward: CCGCCATGGGAATGGTAATGGCAGCGGTAATTCATAAG

Reverse: CGCTCTAGACTACGCTTGCGGAAGCAAAACAAT

The forward and reverse primers included a NcoI and XbaI restriction site (underlined), respectively. The PCR product was digested with NcoI and XbaI and cloned into

pTrc99A (Amp^R). This construct was constructed by Dr. Yipeng Wang (Dr. Bennett's lab, Rice University).

2.2 Culture medium

Chemicals used in the following procedures were purchased from Sigma-Aldrich (St. Louis, MO), unless denoted otherwise.

2.2.1 Cultivation medium

Luria-Bertani (LB) broth medium contained 10 g/L tryptone (Amresco, Solon, OH), 5 g/L yeast extract (BD Biosciences, Franklin Lakes, NJ) and 10 g/L NaCl. The LB medium was autoclaved at 121°C for 20-35 minutes, depending upon the volume of liquid being autoclaved. After autoclaving, glucose, antibiotics, and other various compounds were added aseptically as need be in accordance with each experiment.

For aerobic and anaerobic (S)-2-chloropropionate production shake flask experiments, antibiotics were added at concentrations of 100 mg/L ampicillin and 35 mg/L chloramphenicol for strain selection and plasmid stability. The LB medium used was supplemented with 1% glucose as specified in experimental procedures. (Kurata 2005, Kurata 2008)

For aerobic poly(3-hydroxybutyrate) (PHB) production experiments, a cocktail mixture of ampicillin, carbenicillin, and oxacillin was added at a concentration of 200 mg/L. The LB medium used was supplemented with 2% glucose as specified in experimental procedures. (Sanchez 2006)

2.2.2 Defined M9 medium without ammonium chloride

M9 minimal growth medium contains 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl. $CaCl_2$, $MgSO_4$, and Vitamin B1 (thiamine) were added to the medium after autoclaving to final concentrations of 1 mM, 0.1 mM, and 1 μg/mL, respectively. The medium was supplemented with 100 mg/L ampicillin, 35 mg/L chloramphenicol, and 2% glucose unless indicated otherwise.

2.2.3 Agar plates

Cells were grown on agar plates for strain selection and maintained for short term storage. Plates were prepared using LB and 15 g/L of technical agar. The mixture was autoclaved and allowed to cool to approximately 55 °C. Upon cooling, antibiotics were added as specified by each individual experiment prior to pouring approximately 25 mL of the agar mixture into each plate.

2.2.4 Storage medium

Glycerol stocks were used to maintain strain viability during long term storage. Stocks were prepared using 300 μL of glycerol freezing solution (50% glycerol, filter sterilized) and 700 μL of overnight culture. The mixture was vortexed and left at room temperature for about 30 minutes to allow cells to acclimate to the new environment prior to storage at -80 °C.

2.3. Cell Cultivation

2.3.1(S)-2-chloropropionate production experiments

Cultures for (S)-2-chloroproprionate production experiments were performed using 500 mL shake flasks containing 150 mL of LB media containing the appropriate antibiotics. No glucose was present in the medium a carbon source. Overnight precultures were prepared by inoculating a single colony into 3 mL LB supplemented with appropriate antibiotics, 1% glucose content, and incubated overnight at 37 °C and 250 rpm. Cultures were inoculated with 1.5 mL (1% v/v) of the overnight culture. After inoculation cells were grown in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 30 °C and 250 rpm. When the optical density at 600 nm reached approximately 0.5, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM to the medium. Cells were cultivated in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT)at 30 °C and 250 rpm for approximately 18 hours in the presence of IPTG prior to being harvested for the production of (S)-2-chloroproprionate under aerobic and anaerobic conditions.

2.3.1.1 Aerobic shake flask experiments

Aerobic shake flask experiments were performed using 250 mL shake flasks containing 20 mL of defined M9 media. 2-chloroacrylate was added to the medium to a final concentration of 50 mM and the mixture was pH adjusted to 7.0 with 6 N HCl. Cultured cells were harvested by centrifugation, washed, and resuspended in the reaction medium to a final concentration of approximately 10 OD_{600} to monitor the degradation of 2-chloroacrylate into (S)-2-chloropropionate. A volume of 20 mL of resuspended culture

was transferred aseptically to each flask. Flasks were capped with foam stoppers. The reaction was carried out in in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 37 °C and 250 rpm. Samples of the cultures were taken at 0 and 6 hours and analyzed for (S)-2-chloropropionate and extracellular metabolite production.

2.3.1.2 Anaerobic shake tube experiments

Anaerobic shake tube experiments were performed using 15 mL amber-colored glass tubes containing 14.5 mL of defined M9 media. 2-chloroacrylate was added to the medium to a final concentration of 50 mM and the mixture was pH adjusted to 7.0 with 6 N HCl. Cultured cells were harvested by centrifugation, washed, and resuspended in the reaction medium to a final concentration of approximately 10 OD_{600} to monitor the degradation of 2-chloroacrylate into (S)-2-chloropropionate. A volume of 14.5 mL of resuspended culture was transferred aseptically to each tube to ensure anaerobic conditions. Tubes were capped with a rubber septum. The reaction was carried out in in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 37 °C and 250 rpm. Samples of the cultures were taken at 0 and 6 hours and analyzed for (S)-2-chloropropionate and extracellular metabolite production.

2.3.2 Poly(3-hydroxybutyrate) production experiments

2.3.2.1 Aerobic shake flask experiments

Overnight precultures were prepared by inoculating a single colony into 5 mL LB supplemented with appropriate antibiotics with no glucose content and incubated overnight at 37 °C and 250 rpm. Cultures were inoculated with 1% v/v of the overnight

preculture (e.g. 0.5 mL overnight preculture into a 250 mL flask containing 50 mL LB medium). Aerobic shake flask experiments were performed using 250 mL shake flasks containing 50 mL of LB media. The LB media was supplemented with a cocktail mixture of ampicillin, carbenicillin, and oxacillin to a final concentration of 200 mg/L and 2% glucose. Flasks were capped with foam stoppers and grown in in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 37 °C and 250 rpm. Samples of the culture were taken at 24 and 31 hours and analyzed for poly(3-hydroxybutyrate) and extracellular metabolite production.

2.4 Analytical Techniques

2.4.1 Cell Density

Cell density (OD) was taken at 600nm in a DU 800 spectrophotometer (Beckman Coulter, Irving, TX). Culture samples were diluted, as appropriate, with LB medium to maintain readings in the linear range of the spectrophotometer (0.1-0.4 OD_{600}).

2.4.2 Analysis of extracellular metabolites

In order to quantify extracellular metabolites, 1 mL of the fermentation broth was sampled and centrifuged at 13,000 g for 3 minutes in a microcentrifuge. The supernatant was filtered through a 0.2 μm PTFE syringe filter and stored frozen for HPLC analysis. A Shimadzu-10A HPLC System (Shimadzu Scientific Instruments, Columbia, MD) equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A), and a differential refractive index detector, RI, (Waters, Milford, MA) was used to detect extracellular metabolites such as glucose, succinate,

lactate, and acetate, among others. A solution of 2.5 mM H_2SO_4 was used as the mobile phase and operated at a0.5 mL/min flow rate. The column was operated at 55°C.

2.4.3 PHB Extraction

Samples for PHB extraction were withdrawn aseptically after 24 and 31 hours of cultivation. A 20 mL sample was withdrawn from the aerobic shake flasks at each timepoint, washed three times with 0.15 N NaCl and resuspended in 15 mL of 0.15 N NaCl. Resuspended cells were transferred to preweighed and predried glass tubes and dried to constant weight using dry nitrogen. A control tube containing 15 mL of 0.15 N NaCl was concurrently dried to constant weight and subtracted from the sample's measured dry cell weight. Samples of PHB-containing dry cell mass were digested in 2 mL of concentrated H_2SO_4 for 45 minutes at 100°C on a heating block. After cooling on ice, 10 mL of 0.014 N H_2SO_4 was added to the tubes and vortexed to ensure rapid mixing. Extracted samples were diluted once more with 0.014 N H_2SO_4 to a final dilution factor ranging from 12-72, as needed. (Sanchez, 2006)

2.4.4 Analysis of PHB production

PHB production was quantified using a Shimadzu-10A HPLC System (Shimadzu Scientific Instruments, Columbia, MD) equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A), and a differential refractive index detector, RI, (Waters, Milford, MA). Approximately 1 mL of the diluted extract was centrifuged at 13,000 g for 3 minutes in a microcentrifuge. The supernatant was filtered through a 0.2 μm PTFE syringe filter and stored chilled for

HPLC analysis. The mobile phase used was a 2.5 mM H_2SO_4 solution at a flow rate of 0.6 mL/min. The column was operated at 55°C.

The amount of PHB produced was calculated based upon a method developed by Karr et al. (Karr et al., 1983). The absorbance of crotonic acid was measured at 210 nm with a retention time of 25.2 minutes. The amount of crotonic acid derived from PHB was calculated using a known standard calibration curve of crotonic acid. PHB content was estimated based upon the amount of crotonic acid detected using the previously determined conversion rate of PHB to crotonic acid of 1.81, as described by Taguchi et al. (Taguchi et al., 2001).

2.5 Cell Transformation

Chemical cell transformations were performed based upon the rubidium chloride method by New England Biolabs, Inc. Chemically competent cells were prepared by inoculating 5 mL of LB medium, supplemented with the appropriate antibiotics as specified in each experiment, with a single colony from an LB rich plate. Cells were grown overnight in in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 37°C and 250 rpm. A volume of 0.5 mL of the overnight culture was used to inoculate 50 mL of LB supplemented with 20 mM $MgSO_4$ and appropriate antibiotics. Subcultures were grown to OD_{600} values of approximately 0.4-0.6 and centrifuged at 5,000 rpm for 5 minutes at 4 °C. The pellet was gently resuspended in 20 mL ice cold TFBI (30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, and 15% glycerol, filter sterilized and pH adjusted to 5.8 with acetic acid), incubated on ice for 5 minutes, and centrifuged once more at 5,000 rpm for 5 minutes at 4 °C. The pellet was

gently resuspended in 2 mL ice cold TFBII (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, and 15% glycerol, filter sterilized and pH adjusted to 6.5 with KOH) and incubated on ice for 15-60 minutes. Aliquots were prepared by pipetting 100 µL of the resuspended cells into 1.5 mL sterile centrifuge tubes and stored at -80°C.

Cells were transformed by taking an aliquot of chemically competent cells, thawing on ice, adding 1-1.5 µL of DNA, and incubating on ice for 30-60 minutes. The mixture was heat shocked for 45 seconds at 37°C and incubated on ice for 2 minutes. Transformed cell suspensions were diluted into 1.4 mL LB with no antibiotics to allow for recovery and phenotypic expression. Following cultivation in in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 37 °C and 250 rpm for 40-60 minutes, transformed cells were plated on selective medium and grown overnight at 37°C.

Chapter 3

3. Effects of Pyridine Nucleotide Transhydrogenases on the Production of (S)-2-chloropropionate in *Escherichia coli*

3.1 Abstract

The syntheses of several industrially useful compounds are cofactor-dependent. Biocatalysis has emerged as a valuable tool for large-scale, industrial production processes as it is a cost-effective means of production, acting as both a catalyst and cofactor regenerator. The reducing equivalent NADPH is required in several enzymatic reactions leading up to the synthesis of high-value compounds such as polymers, chiral alcohols, and antibiotics, to name a few. However, it is a highly costly compound and its intracellular availability can oftentimes act as a limiting factor in product yield. This study focuses on the genetic manipulation of a whole-cell system using the two transhydrogenase isoforms *pntAB* and *udhA*. *Escherichia coli* strains are engineered to evaluate the effects of the two transhydrogenases on NADPH availability in order to increase the NADPH pool for improved production of products that require NADPH in their biosynthesis. Recombinant *E. coli* strains containing *pntAB* and/or *udhA* deletion and/or *pntAB* or *udhA* overexpression were constructed. Genetic manipulations and subsequent effects on NADPH availability were assessed using the following model system: the production of (S)-2-chloropropionate catalyzed by the NADPH dependent 2-haloacrylate reductase (CAA43) under both aerobic and anaerobic conditions. Results suggest that the presence of *udhA* increases product yield and NADPH availability while the presence of *pntAB* has the opposite effect. A maximum product yield of 1.4 mole

product/mole glucose was achieved aerobically in a *pntAB*-deletion strain with *udhA* overexpression, a 150% improvement over the wild-type control strain.

3.2 Introduction

As the fields of genetic and metabolic engineering have advanced over the years so have their applications in both industrial settings and academic institutions. In particular, the implementation of biocatalysis for industrial purposes has gained considerable ground. Biocatalysts are widely accepted as excellent means for large-scale, industrial chemical processes. They exhibit regio- and sterio- specificities and call for mild culture conditions (Endo, 2001).

Currently, most genetic and metabolic engineering studies place an emphasis on manipulating enzyme levels via the addition, amplification, and/or deletion of pathways. The majority of biocatalysts in use are cofactor-independent ones that carry out rather elementary chemical reactions, such as hydrolases (Zhao, 2003). Cofactor-dependent enzymes, on the other hand, are capable of carrying out more complex chemistry and are known to catalyze an assortment of industrially-relevant reactions. Oxidoreductases and transferases are two examples of industrially useful compounds, as they are able to catalyze several regio- and stereo- selective reactions for the synthesis of high purity chiral compounds. However, the syntheses of these compounds are dependent upon low molecular weight, non-protein compounds known as cofactors, which play a role in several biochemical reactions. Subsequently, cofactor level is an additional control parameter to consider and acts as supplemental tool when attempting to study and achieve specific metabolic engineering goals.

Some cofactors, like biotin and pyridoxal phosphate, are self-regenerating. Others, like pyridine nucleotides and nucleoside triphosphates, act as co-substrates and transfer agents that accept or donate functional groups (hydrides, phosphates, etc.) in an enzymatic reaction (Chenault, 1988). Cofactors like these must be provided in stoichiometric quantities. Reducing equivalent cofactors, like NADPH, are needed in several enzymatic reactions leading up to the synthesis of high-value compounds. Chiral compounds, for instance, utilize NADPH and are common intermediates used in the production of pharmaceutical products, like the stereoselective synthesis of statins, a cholesterol-lowering drug (Panke, 2005). Unfortunately, in spite of their utility, these cofactors are costly. Their use in large-scale industrial settings is precluded by their high costs (Table 3.1). In order to make these processes economically feasible, an effective regeneration system is needed.

Table 3-1. Average cost per gram mole of common cofactors (adapted from Chenault, 1988).
Key: a) Kyowa Hakko Kogyo Co., Ltd.
b) Sigma Chemical Co.
c) United States Biochemical Corp.

Cofactor	$/g mol
Acetyl CoA	1,300,000 [c]
CoA	249,000 [c]
ATP	220 [a]
NAD	710 [a]
NADH	3,100 [a]
NADP	25,800 [b]
NADPH	216,000 [b]

Many cofactor regeneration systems have been studied in the past. Four broad categories of pyridine cofactor regeneration have been previously described by Chenault et al. (1987) and are as follows: biological, enzymatic, electrochemical, and chemical/photochemical. Chemical regeneration methods are rather straightforward,

commercially available, and cost effective. However, they are marked by limited compatibility with enzymatic systems, low product yield, and slow rates. Electrochemical and photochemical regeneration methods utilize electrical or light energy as a driving force in the cofactor regeneration step, but again are characterized by limited compatibility with enzymatic systems, low selectivity, and poor selectivity. Enzymatic regeneration methods are further delineated and divided into two subcategories: substrate-coupled and enzyme-coupled. In substrate-coupled enzyme regeneration systems, a single enzyme uses both the reduced and oxidized forms of a cofactor alongside two substrates and catalyzes both the main reaction of interest as well as the regeneration reaction. Whereas in an enzyme-coupled regeneration system, two different enzymes and substrates are used, catalyzing the reaction of interest and the regeneration reaction (Wichmann, 2005). The benefits of these enzymatic regeneration systems are their high selectivity, high compatibility with enzyme-catalyzed synthesis, and high reaction rates. However, the use of isolated enzymes is not economically feasible, especially when multiple enzymatic steps are needed or when reactions call for large quantities of enzymes. This method is quite costly, as it requires protein purification, external cofactor addition, and/or external co-substrate addition. A more viable and cost-effective alternative is a biological regeneration method that utilizes whole cells or organelles that possess the desired enzyme activity. Whole-cell systems present a more economically-feasible approach because they are, in essence, self-assembling processes. All of the required enzymes are produced by the organism by natural means or by heterologous expression (Schmid, 2001).

Redox reactions have significant commercial utility, as they play a key role in the synthesis of industrially relevant compounds. These reactions require intermediates and cofactors produced via cellular central metabolism. Host organisms rely upon over 1,000 anabolic reactions when synthesizing individual components essential to overall cell function and require cofactor pairs for product formation (Forster 2003). Cofactors such as ATP, NADP, and NADPH, in particular, are of enormous importance for the synthesis of compounds such as antibiotics (Gunnarsson 2004), biopolymers (Sanchez 2006, Wong 2008), and chiral alcohols (Hummel 1997), among others.

Cofactor pairs like NAD/NADP and NADH/NADPH are essential in these processes, as they are capable of reversible transformation between oxidized and reduced states and shuttle electrons in oxidation and reduction reactions. However, because of demanding parameters such as expensive production costs and high cellular demand for reducing equivalents, industrial applications typically utilize whole-cell fermentation systems. As mentioned previously, whole-cell systems offer a viable, cost-effective approach for supplying and regenerating cofactors, such as the reducing equivalent NADPH. However, in spite of their utility, inherent regeneration rates can be a limiting factor for attaining high product yields and productivity (Gunnarsson 2004, San 2002). Additionally, when applying metabolic engineering design strategies, care must be taken to ensure that cofactors are supplied in stoichiometric quantities that allow for regulated, balanced growth.

The oxidative branch of the pentose phosphate pathway (PP) and isocitrate dehydrogenase of the tricarboxylic acid cycle (TCA) cycle are the main two sources of NADPH (Fig. 3-1). The pentose phosphate pathway is responsible for regenerating the

majority of NADPH from $NADP^+$, where two units of the reduced cofactor are produced per glucose (Gunnarsson, 2004). This work focuses on the application of rational genetic modifications using pathway and cofactor engineering to manipulate and characterize NADPH availability by determining product yields of cofactor-dependent products in *Escherichia coli*. This objective is achieved using classic metabolic engineering strategies such as overexpression and deletion of specific enzymes, wherein whole-cell fermentation systems are implemented to allow for more robust and cost-effective compound synthesis. Cofactor constraints are taken into consideration in efforts to attain high product yields and productivities when catalyzing the substrate to product conversions.

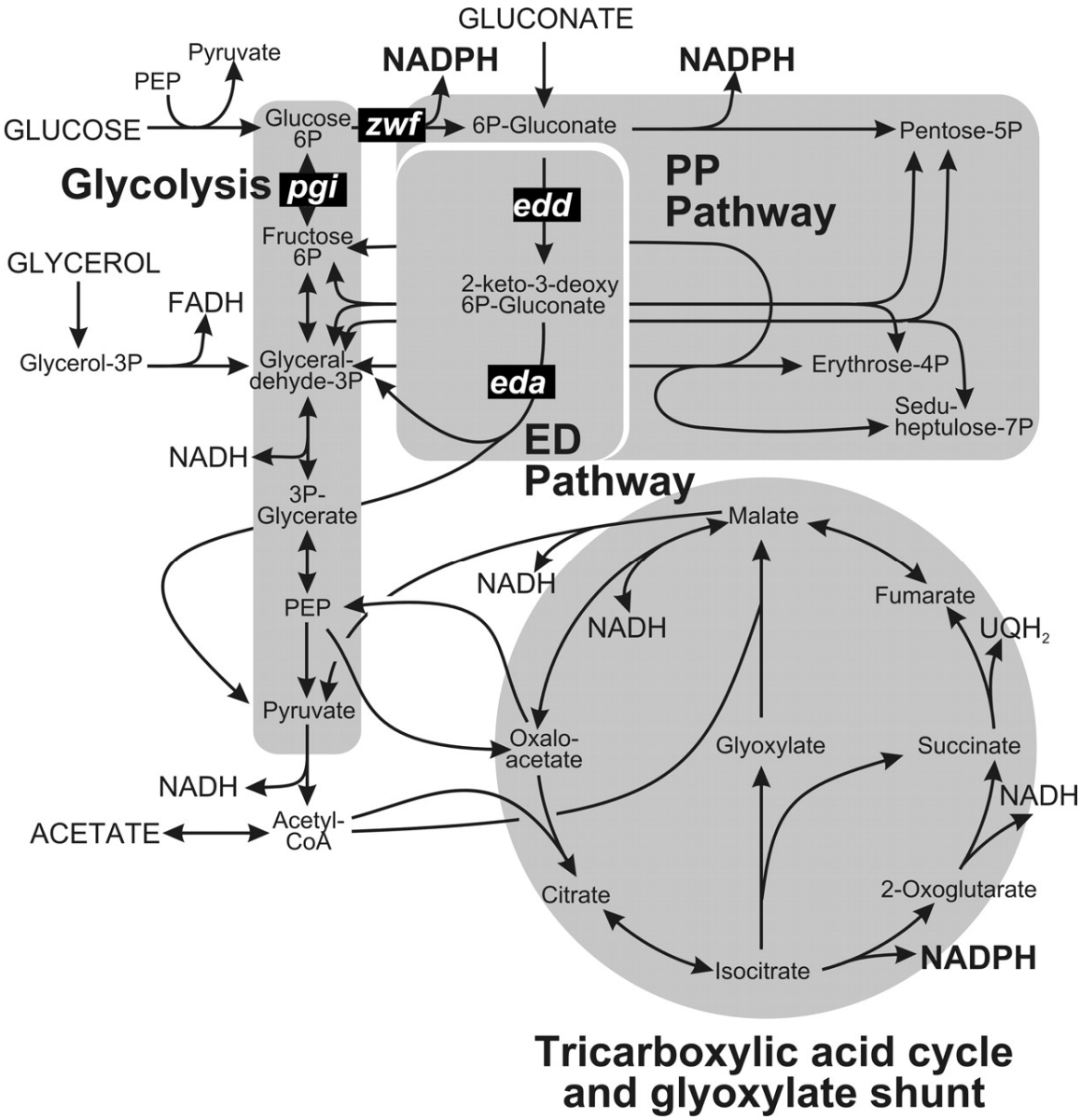

Figure 3-1. Central carbon metabolic pathway of *E. coli*. Primary NADPH-generating reactions are the oxidative step of the pentose phosphate (PP) pathway and the isocitrate dehydrogenase step in the TCA cycle. Adapted from Sauer et al., 2004.

Past studies have manipulated NADPH levels in order to assess productivity. Lee et al. (2009) investigated cofactor effects on the production of thymidine, a commercially-relevant precursor commonly used in the synthesis of antiviral drugs, by overexpressing NADK, an intracellular NAD+ and NADP+ concentration regulator. The

mutant strain increased NADPH levels by 1.3 fold and decreased NADP+ levels by 10%. The subsequent increase in the NADPH/NADP+ ratio resulted in a 1.2 fold increase in thymidine production. Moreira dos Santos et al. (2004) added an additional source of NADPH into cells by overexpressing the malic enzyme in *Saccharomyces cerevisiae*, resulting in decreased pentose phosphate pathway flux and a net transhydrogenase effect, indicating potential for increasing NADPH production capacity. Verho et al. (2003) facilitated NADPH regeneration in *S. cerevisae* by overexpressing the fungal $NADP^+$-dependent D-glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH) from *Kluyveromyces lactis*, which was previously discovered to regenerate NADPH (Verho, 2002). NADPH levels have also been manipulated in *E. coli* by knocking out the phosphorglucosisomerase (*pgi*) gene. Deletion of the *pgi* gene forces carbon flow through the pentose pathway, resulting in the overproduction of NADPH. However, this overproduction was found to have a detrimental effect on cell growth (Canonaco 2001, Hanson 1980, Kabir 2003). Lee et al. (2007) observed heightened production of ε-caprolactone in recombinant *E. coli* strains expressing cyclohexane monooxygenase (CHMO) and the NADPH regenerating enzyme, glucose 6-phosphate dehydrogenase, encoded by the *zwf* gene in *E. coli*. They achieved maximal ε-caprolactone concentrations of 15.3 g/L, a 39% improvement in comparison to the control strain (Lee, 2007). Sanchez et al. (2006) increased NADPH availability in *E. coli* by overexpressing the soluble pyridine nucleotide transhydrogenase, *udhA*, resulting in high yield and productivity of the biodegradable polymer, poly(3-hydroxybutyrate) (PHB). They reported a significant increase in NADPH-dependent PHB production with a *udhA* overexpression strain.

Thus far, two types of pyridine nucleotide transhydrogenases have been documented in living organisms. The energy-linked transhydrogenase, *PntAB* is a membrane-bound, proton-translocating transhydrogenase located in the mitochondria of heterotrophic and photosynthesizing bacteria. While *UdhA* is a soluble, energy-independent transhydrogenase found in some heterotrophic bacteria (Sauer 2004, Voordouw 1983). Typically, only one transhydrogenase isoform or none altogether is inherently found in microorganisms. The exception to this rule is the Enterobacteriaceae family, which encompasses *E. coli* and contains both transhydrogenase isoforms: *PntAB* and *UdhA* (Boonstra 1999, Clarke 1986, Sauer 2004). These two isoforms are theorized to have a large role in maintaining redox balance within microorganisms.

However, they are not thoroughly studied and their physiological role is not clearly understood. Limited information on pyridine nucleotide transhydrogenases in *E. coli* is at our disposal. Additionally, a consensus has yet to be reached regarding their unique roles and direction of the reaction they catalyze (Canonaco 2001, Chin 2008, Hansen 2005, Hanson 1980, Hoek 1988, Jackson 1998, Sanchez 2006, Sauer 2004). The common line of thought marks the oxidative steps of the pentose phosphate pathway and the isocitrate dehydrogenase steps of the TCA cycle as the primary sources for generating NADPH (Sauer 2004). Nevertheless, pyridine nucleotide transhydrogenases are also speculated to play an important role in modulating and maintaining the availability of reducing equivalents, like NADPH, within the cell. Yet, as mentioned previously, since the discovery of transhydrogenases, their physiological role has yet to be clearly and definitively understood.

An early study by Hanson and Rose in 1980 created an insertion mutation in the pnt locus of *E. coli* cells and concluded that the transhydrogenase is not necessary for the production of NADPH or ATP in *E. coli*, as no significant changes in growth rate were observed when grown using a variety carbon sources. In the same study, Hanson and Rose also constructed pnt mutant strains in both a glucose-6-phosphate-1-dehydrogenase (*zwf*) and a phosphoglucose isomerase (*pgi*) mutant background to examine the importance of the transhydrogenase in NADPH production. *Zwf* is the first enzyme in the NADPH-generating pentose phosphate pathway while *pgi* is an NADPH-dependent gene involved in glycolosis. When a single *zwf* mutant and a single *pgi* mutant were constructed, the *pgi* mutant exhibited lowered growth rate in comparison to the wild type strain while the *zwf* mutant's growth rate was not significantly affected. When the pnt mutation was introduced into these mutant backgrounds, the *zwf* mutant strain was found to have a significantly lower growth rate while the *pgi* mutant strain remained unchanged. The observed trends in growth rate suggest that *pntAB* is primarily involved in supplying NADPH. This notion that *pntAB* acts as a major source for NADPH generation was also supported by studies by Sauer et al. (2004). They reported that 35-45% of NADPH produced during aerobic growth on glucose was from *pntAB*, compared to 35-45% and 20-25% via the pentose phosphate pathway and isocitrate dehydrogenase, respectively.

The soluble transhydrogenase, *udhA*, was previously reported to have a prominent role under conditions where there is significant NADPH overproduction (Canonaco 2001, Sauer 2004). Canonaco et al. (2001) examined the effects of *udhA* overexpression in *pgi* mutant *E. coli* strains. As mentioned previously, deletion of the *pgi* gene blocks glycolytic breakdown of glucose and forces carbon flow through the pentose pathway,

resulting in excess NADPH production and a decline in NADH availability (Canonaco 2001, Hanson 1980, Kabir 2003). *Pgi* mutant strains are also marked by significantly reduced growth rates. When coupled with *udhA* overexpression, Canonaco et al. reported partial growth rate restoration by approximately 25%. This finding supports the hypothesis that the kinetic limitation in glucose catabolism in *pgi* mutants is partially due to insufficient reoxidation of NADPH. Thus, the apparent function of *udhA* is the reduction of NAD+ to NADH using NADPH as an electron donor. Complete recovery of growth was not observed in *pgi* mutants because the pentose pathway has limited capacity in comparison to the glycolosis-TCA cycle pathway in terms of cell growth (Canonaco, 2001). Another study by Hansen and Schonheit (2005) examined a double mutant strain of *E. coli* lacking both the *pgi* and *udhA* genes. This *pgi-udhA* mutant strain was unable to grow using glucose as its sole energy source, offering further support for the hypothesis that *udhA* is essential in the reoxidation of NADPH by reducing NAD+.

As of now, only a handful of articles have examined the function of pyridine nucleotide transhydrogenases and their effects on the synthesis of cofactor-dependent products. Chin et al. (2009) investigated the effects of transhydrogenase deletion and overexpression mutant strains on NADPH-dependent xylitol yield. They reported no significant improvement in xylitol yield when *pntAB* and *udhA* were overexpressed in two separate strains with xylose reductase from *Candida boidinii*. In fact, they noted an unexpected decrease in xylitol yield when *pntAB* was overexpressed. Furthermore, *pntAB*, *udhA*, and *pntAB-udhA* deletions had no apparent effect on xylitol yield. This finding is in contrast to a study performed by Weckbecker and Hummel (2004). They examined NADPH-dependent production of (R)-phenylethanol from acetophenone in

alcohol dehydrogenase (ADH) and formate dehydrogenase (FDH) overexpressing *E. coli* cells. The NADP+-dependent ADH from *Lactobacillus kefir* reduces acetophenone and was coupled with FDH from *Candida boidinii*, as it is an effective NADH regenerator. (R)-phenylethanol production was compared in an ADH-FDH overexpression strain and an ADH-FDH overexpression strain containing a *pntAB* overexpression cassette, which essentially acts as an NADPH and NAD+ regenerator (see Fig. 3-2). (R)-phenylethanol production increased from 19% to 66% in the *pntAB*-overexpressing strain, supporting the notion that NADPH was the limiting factor in the reaction and that *pntAB* regenerated NADPH. An additional study performed by Sanchez et al. (2006) overexpressed the soluble transhydrogenase, *udhA*, in *E. coli* and examined its subsequent effects on the yield and productivity of NADPH-dependent poly(3-hydroxybutyrate) (PHB). This setup allowed them to study the effect of *udhA* when NADPH is in high demand. They noted a significant increase in PHB yield and concentration, increasing from 49 to 66% and 3.52 to 6.42 g/L, respectively. This finding suggests that, in circumstances when there is significant NADPH drain such as in the synthesis of PHB, *udhA* favors the direction of NADPH production. Thus, overexpression results in a greater NADPH pool (Chin 2009, Sanchez 2006). A recent study by Lee et al. (2010) examined NADPH-dependent thymidine production by constructing a *udhA*-overexpressing strain in a *pgi* deletion background. When compared to the control strain, thymidine production increased 2-fold with *udhA* overexpression. Furthermore, they measured expression levels of redox-related enzymes in *pgi* mutant strains with either *udhA* or *yfjB* (NAD+ kinase; NADK) overexpression. NADK generates a NADP+ pool from NAD+ in *E. coli*. They found that NADK overexpression increased intracellular redox equivalent levels significantly,

accompanied by higher transhydrogenase expression levels (*pntAB* and *udhA*) in order to maintain intracellular redox balance. Both NADPH and NADP+ levels were increased in this instance. Contrastingly, in *udhA* overexpression, intracellular redox equivalent levels were only moderately increased and were accompanied by elevated NADPH and lower NADP+ levels. In this respect, *udhA* overexpression had a significant effect on the intracellular NADPH/NADP+ ratio, contributing to enhanced thymidine production. The authors suggest that *udhA* elevates NADPH availability in an indirect manner by reducing the metabolic burden on the cell and modulating a favorable NADH and NADPH distribution.

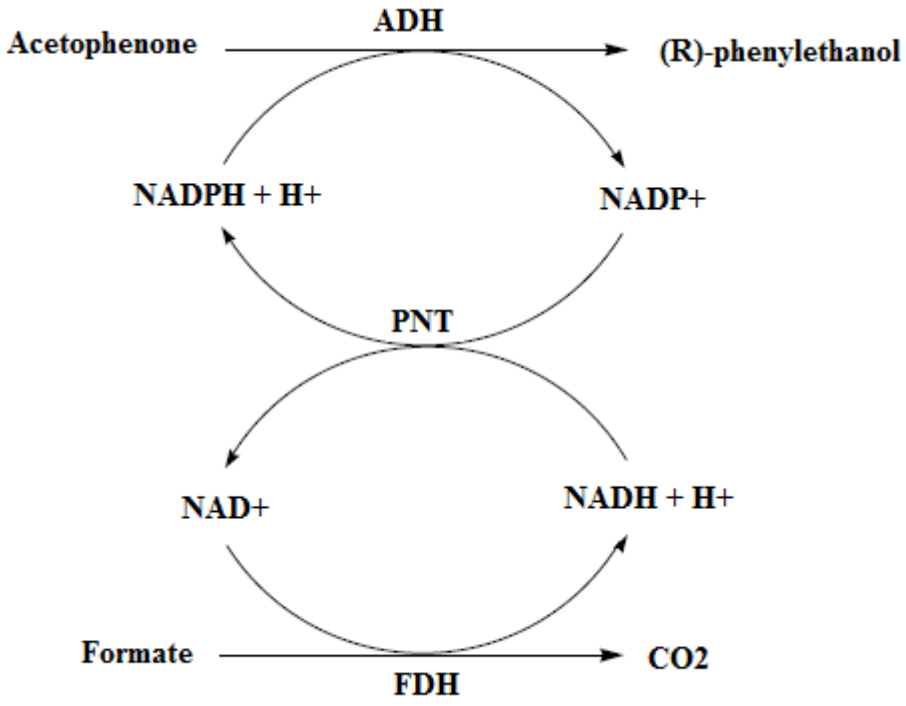

Figure 3-2. An (R)-phenylethanol production system coupling alcohol dehydrogenase (ADH), formate dehydrogenase (FDH), and the membrane-bound transhydrogenase *pntAB* for the regeneration of reducing equivalents. Adapted from Weckbecker and Hummel, 2004.

Evidently, we have yet to achieve a thorough understanding in regards to the roles associated with the two transhydrogenase isoforms in *E. coli*. As a whole, literature sources suggest that *pntAB* drives the transfer of reducing power from NADH to NADP+ while *udhA* favors the transfer of reducing power from NADPH to NAD+ (Fig. 3-3). Yet, reports suggest that both isoforms are capable of catalyzing the opposite reaction depending upon culture conditions, environmental perturbations, and genotype. Additionally, as the two isoforms are involved in maintaining the redox balance between NADPH and NADH, the deletion and/or overexpression of one or the other has been hypothesized to relieve burdens/limitations posed upon cofactors, resulting in higher yields in NADPH-dependent product synthesis reactions. In order to gain a better understanding about the roles and underlying mechanisms of the two isoforms on cofactor availability to enhance NADPH-dependent productivity, additional research is needed.

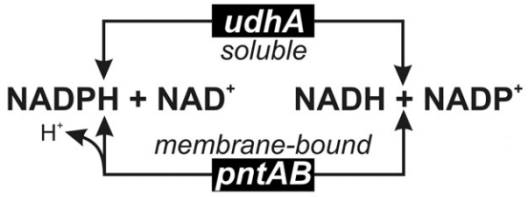

Figure 3-3. Proposed mechanism for NADPH shuffling by the two transhydrogenase isoforms *udhA* and *pntAB*. Adpated from Sauer et al. (2003).

In this study, we examine the effects of two transhydrogenases (*PntAB* and *UdhA*) on NADPH availability. *PntAB*, a membrane-bound, proton-translocating transhydrogenase, and *UdhA*, a soluble, energy-independent transhydrogenase, function

in NADPH metabolism of *E. coli* (Sauer 2004). These two transhydrogenease isoforms transfer electrons between NAD and NADP in accordance with the following equation:

$$NADH + NADP^{+} \leftrightarrow NAD^{+} + NADPH.$$

Genetic manipulations and subsequent effects on NADPH availability were assessed using the following model system: the production of (S)-2-chloropropionate catalyzed by the NADPH dependent 2-haloacrylate reductase (CAA43). The CAA43 gene encoding the enzyme 2-haloacrylate reductase, which catalyzes the NADPH-dependent reduction of the carbon-carbon double bond in 2-chloroacrylate, was first discovered by Kurata et al. (2005) and has been cloned and expressed in *Escherichia coli* from its native host *Burkholderia* sp. WS, a soil bacterium (Kurata 2005, 2008). The production of (S)-2-chloropropionate from 2-chloroacrylate was chosen as a model system for monitoring NADPH availability because 2-haloacrylate reductase, the enzyme catalyzing the one-step conversion to (S)-2-chloropropionate in the synthesis pathway (Fig. 3-4), requires NADPH as a cofactor. In the following studies, *E. coli* strains were transformed with the appropriate plasmids encoding transhydrogenase gene deletions and/or overexpressions, cultured, and assayed for product formation and NADPH availability.

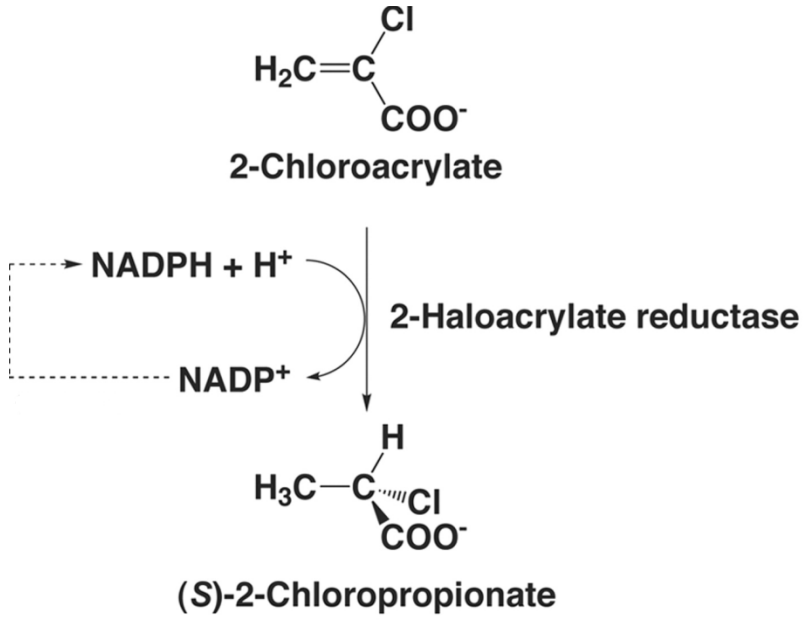

Figure 3-4. Pathway for the degradation of 2-chloroacrylate to (S)-2-chloropropionate, catalyzed by the enzyme 2-haloacrylate reductase (CAA43). Adapted from Kurata et al., 2005.

3.3 Materials and methods

3.3.1 Bacterial strains and plasmids

The strains and plasmids used in this study are described in Table 3-2, below. Wild type (MG1655), single *udhA* (MBS601) and *pntAB* (MBS602) transhydrogenase mutants, and a double transhydrogenase mutant (MBS603) were transformed with plasmids pCAA43, containing the 2-haloacrylate reductase gene, and either p*PNTAB* carrying the *pntAB* gene or pUDHAC carrying the *udhA* gene. pDHC29 was used as a control. Construction of these strains is described in detail in section 2.1.2 of the Materials and Methods chapter.

Table 3-2. List of *E. coli* strains and plasmids used in this study.

	Genotype	References
Strain		
MG1655	Wild type *E. coli* ($F^- \lambda^-$)	ATCC 47076
MBS601	MG1655 Δ*udhA*	This study
MBS602	MG1655 Δ*pntAB*	This study
MBS603	MG1655 Δ*pntAB* Δ*udhA*	This study
Plasmids		
pDHC29	Cloning vector, Cm^R	(Phillips et al. 2000)
pCAA43	2-Haloacrylate reductase gene from *Burkholderia sp*. in pTrc99A, Amp^R	(Kurata et al. 2005)
p*PNTAB*	Proton-translocating pyridine nucleotide transhydrogenase gene *(pntAB)* from *E. coli*, in pDHC29, Cm^R	This study
pUDHAC	Soluble pyridine nucleotide transhydrogenase gene (*udhA*) from *E. coli* in pDHC29, Cm^R	This study

3.3.2 Cultivation medium

Chemicals used in the following procedures were purchased from Sigma-Aldrich (St. Louis, MO), unless denoted otherwise.

3.3.2.1 LB medium

Luria-Bertani (LB) broth medium contained 10 g/L tryptone (Amresco, Solon, OH), 5 g/L yeast extract (BD Biosciences, Franklin Lakes, NJ) and 10 g/L NaCl. The LB medium was autoclaved at 121°C for 20-35 minutes, depending upon the volume of liquid being autoclaved. After autoclaving, glucose, antibiotics, and other various compounds were added aseptically as need be in accordance with each experiment.

For aerobic and anaerobic (S)-2-chloropropionate production shake flask experiments, antibiotics were added at concentrations of 100 mg/L ampicillin and 35 mg/L chloramphenicol for strain selection and plasmid stability. The LB medium used was supplemented with 1% glucose as specified in experimental procedures. (Kurata 2005, Kurata 2008)

3.3.2.2 Defined medium

M9 minimal growth medium contains 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl. $CaCl_2$, $MgSO_4$, and Vitamin B1 (thiamine) were added to the medium after autoclaving to final concentrations of 1 mM, 0.1 mM, and 1 μg/mL, respectively. The medium was supplemented with 100 mg/L ampicillin, 35 mg/L chloramphenicol, and 2% glucose unless indicated otherwise.

3.3.3 Cultivation conditions

3.3.3.1 Cultivation conditions in LB medium

Cultures for (S)-2-chloroproprionate production experiments were performed using 500 mL shake flasks containing 150 mL of LB media containing the appropriate antibiotics. No glucose was present in the medium a carbon source. Cultures were inoculated with 1.5 mL (1% v/v) of the overnight culture. After inoculation cells were grown in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 30 °C and 250 rpm. When the optical density at 600 nm reached approximately 0.5, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM to the medium. Cells were cultivated in an Excella E25 rotary shaker (New Brunswick

Scientific, Enfield, CT)at 30 °C and 250 rpm for approximately 18 hours in the presence of IPTG prior to being harvested for the production of (S)-2-chloroproprionate under aerobic and anaerobic conditions.

3.3.3.2 Cultivation conditions in defined medium

Aerobic shake flask experiments were performed using 250 mL shake flasks containing 20 mL of defined M9 media. 2-chloroacrylate was added to the medium to a final concentration of 50 mM and the mixture was pH adjusted to 7.0 with 6 N HCl. Cultured cells were harvested by centrifugation, washed, and resuspended in the reaction medium to a final concentration of approximately 10 OD_{600} to monitor the degradation of 2-chloroacrylate into (S)-2-chloropropionate. A volume of 20 mL of resuspended culture was transferred aseptically to each flask. Flasks were capped with foam stoppers. The reaction was carried out in in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 37 °C and 250 rpm. Samples of the cultures were taken at 0 and 6 hours and analyzed for (S)-2-chloropropionate and extracellular metabolite production.

Anaerobic shake tube experiments were performed using 15 mL amber-colored glass tubes containing 14.5 mL of defined M9 media. 2-chloroacrylate was added to the medium to a final concentration of 50 mM and the mixture was pH adjusted to 7.0 with 6 N HCl. Cultured cells were harvested by centrifugation, washed, and resuspended in the reaction medium to a final concentration of approximately 10 OD_{600} to monitor the degradation of 2-chloroacrylate into (S)-2-chloropropionate. A volume of 14.5 mL of resuspended culture was transferred aseptically to each tube to ensure anaerobic conditions. Tubes were capped with a rubber septum. The reaction was carried out in in

an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 37 °C and 250 rpm. Samples of the cultures were taken at 0 and 6 hours and analyzed for (S)-2-chloropropionate and extracellular metabolite production.

3.3.4 Inoculum preparation and conditions for (S)-2-chloropropionate experiments

For each experiment, wild-type, single *udhA* and *pntAB* transhydrogenase mutants, and the double transhydrogenase mutant were freshly transformed with the pCAA43 plasmid and appropriate overexpression or control plasmids. A single colony was restreaked onto a plate containing 100 mg/L ampicillin and 35mg/L chloramphenicol. A single colony was transferred into 3 mL LB supplemented with 100 mg/L ampicillin, 35mg/L chloramphenicol, and 1% glucose and grown overnight aerobically at 37°C and 250 rpm in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT). Cells were inoculated at 1% v/v into 500 mL shake flasks containing 150 mL of LB media supplemented with 100 mg/L ampicillin and 35mg/L chloramphenicol. No glucose was present in the medium a carbon source. Flasks were grown aerobically at 37°C and 250 rpm.

3.3.5 Analytical techniques

Cell density (OD) was taken at 600nm in a DU 800 spectrophotometer (Beckman Coulter, Irving, TX). Culture samples were diluted, as appropriate, with LB medium to maintain readings in the linear range of the spectrophotometer (0.1-0.4 OD_{600}).

In order to quantify extracellular metabolites, 1 mL of the fermentation broth was sampled and centrifuged at 13,000 g for 3 minutes in a microcentrifuge. The supernatant

was filtered through a 0.2 μm PTFE syringe filter and stored frozen for HPLC analysis. A Shimadzu-10A HPLC System (Shimadzu Scientific Instruments, Columbia, MD) equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A), and a differential refractive index detector, RI, (Waters, Milford, MA) was used to detect extracellular metabolites such as glucose, succinate, lactate, and acetate, among others. A solution of 2.5 mM H_2SO_4 was used as the mobile phase and operated at a0.5 mL/min flow rate. The column was operated at 55°C.

3.4 Results and discussion

3.4.1 Aerobic (S)-2 chloropropionate production experiment results

Aerobic shake flask experiments were performed with mutant and control strains in defined M9 medium utilizing glucose as the carbon source to evaluate transhydrogenase effects on NADPH availability. Mutant *E. coli* strain cell growth was comparable to the control, indicating limited growth impairment with the exception of the *pntAB* deletion strains (MBS602 pCAA43 pDHC29, MBS602 pCAA32 *udhA*) (Fig. 3-5).

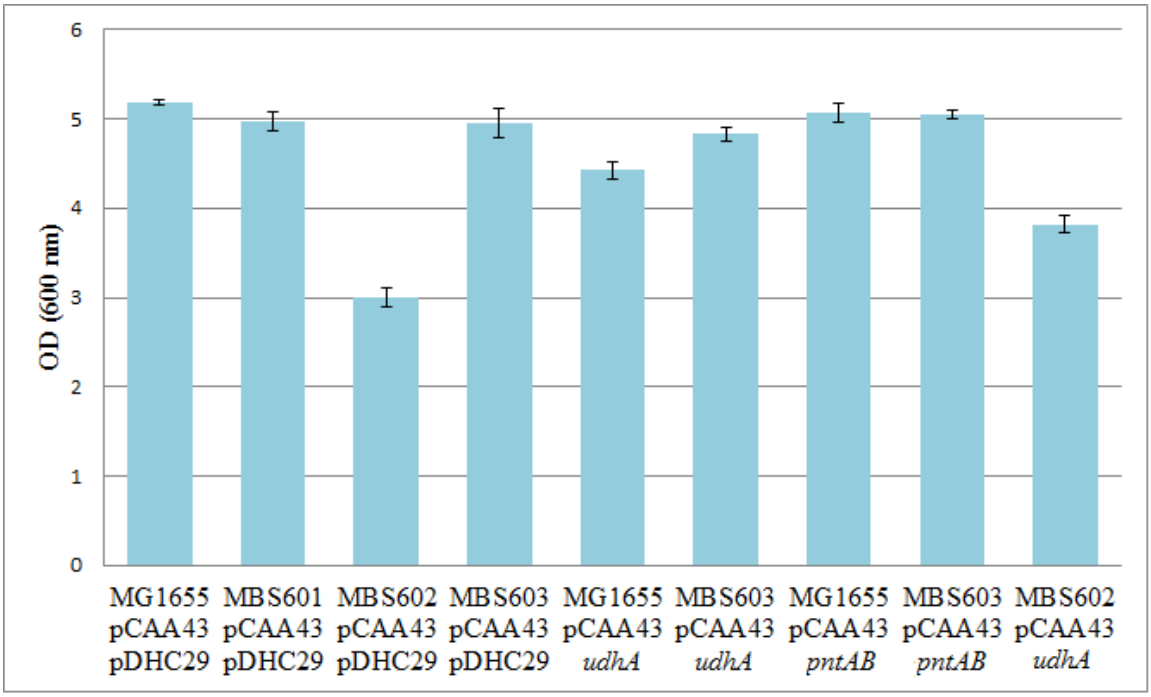

Figure 3-5. Cell growth of control (MG1655 pCAA43 pDHC29) and modified *E. coli* strains (MBS601, Δ*udhA*; MBS602, Δ*pntAB*; MBS603, Δ*pntAB*Δ*udhA*) under aerobic conditions in LB medium with IPTG induction for 18 hours at 30°C and 250 rpm.

Three mutant background strains were evaluated against a control strain (MG1655), each containing the 2-haloacrylate reductase gene (pCAA43) and the empty control vector pDHC29. All three mutant strains exhibited higher product yield than the control (Table 3-3). The mutant strain MBS602 pCAA43 pDHC29 had a significant boost in product yield over the control strain, producing 2.2 times that of the control (1.25 compared to 0.571 mole (S)-2 chloroproprionate/mole glucose). One mole of NADPH is consumed per mole of (S)-2 chloropropionate produced (Fig. 3-4). Therefore, this mutant strain produced 120% more NADPH than the control strain. The mutant strain MBS603 pCAA43 pDHC29 produced 1.41 times that of the control with 42% more NADPH, and the mutant strain MBS601 pCAA43 pDHC29 produced 1.3 times that of the control with 27% more NADPH.

Table 3-3. Production of (S)-2 chloropropionate in shake flasks under aerobic conditions by *E. coli* strains with wild-type and mutant backgrounds in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression and (-) denotes transhydrogenase deletion. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MG1655 pCAA43 pDHC29 *udhA+ pntAB+*	0.5707 ± 0.0163	41.00 ± 1.058
MBS601 pCAA43 pDHC29 *udhA- pntAB+*	0.7271 ± 0.0079	42.23 ± 0.8095
MBS602 pCAA43 pDHC29 *udhA+ pntAB-*	1.252 ± 0.0669	11.59 ± 0.7369
MBS603 pCAA43 pDHC29 *udhA- pntAB-*	0.8075 ± 0.0408	42.71 ± 1.113

When examining the effect of *pntAB* expression in the presence of *udhA* it is apparent that the increasing levels of *pntAB* decrease product yield. Of the three *udhA*-expressing strains shown in Table 3-4, the *pntAB* overexpression (*pntAB*++) strain has the lowest product yield. Both product yield and NADPH levels increase by 10% in the *udhA*+ *pntAB*+ strain (MG1655 pCAA43 pDHC29) in comparison to the *pntAB* overexpression strain. Furthermore, when *pntAB* is removed from the strain altogether (*pntAB*-), both product yield and NADPH levels are maximized in this set of strains. An improvement of 140% and 120% is observed over the *pntAB* overexpression and the *pntAB* expression strains, respectively. The presence of *pntAB* evidently decreases

product yield, with decreasing product yield observed with increasing *pntAB* expression levels.

Table 3-4. Production of (S)-2 chloropropionate in *E. coli* shake flask experiments under aerobic conditions in *udhA*-expressing strains with varying degrees of *pntAB* expression in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression, (-) denotes transhydrogenase deletion, and (++) denotes transhydrogenase overexpression. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MG1655 pCAA43 *pntAB* *udhA+ pntAB++*	0.5191 ± 0.0112	40.54 ± 0.4648
MG1655 pCAA43 pDHC29 *udhA+ pntAB+*	0.5707 ± 0.0163	41.00 ± 1.0581
MBS602 pCAA43 pDHC29 *udhA+ pntAB-*	1.252 ± 0.0669	11.59 ± 0.7369

When examining the effect of *pntAB* expression in the absence of *udhA*, a similar though less pronounced trend is observed (Table 3-5). Product yield and NADPH levels are boosted by 11% upon *pntAB* deletion, increasing from 0.727 mole product/mole glucose to 0.808 mole product/mole glucose. These results mirror those presented earlier, indicating that the presence of *pntAB* decreases product yield. When looking at the effects of *pntAB* deletion in both a *udhA*-deletion (MBS603 pCAA43 pDHC29) and *udhA*-expressing strain (MBS602 pCAA43 pDHC29), we note that a greater product yield is achieved in the *udhA*-expressing strain (1.25 vs. 0.808 mole product/mole glucose), suggesting that the presence of *udhA* may be beneficial in supplying NADPH for the production of (S)-2-chloropropionate.

Table 3-5. Production of (S)-2 chloropropionate in *E. coli* shake flask experiments under aerobic conditions in *udhA*-knockout strains with varying degrees of *pntAB* expression in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression and (-) denotes transhydrogenase deletion. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MBS601 pCAA43 pDHC29 *udhA- pntAB+*	0.7271 ± 0.0079	42.23 ± 0.8095
MBS603 pCAA43 pDHC29 *udhA- pntAB-*	0.8075 ± 0.0408	42.71 ± 1.113

The effects of varying levels of *udhA* expression are elucidated in the following set of strains with *pntAB* deletion presented in Table 3-6. In the absence of both *pntAB* and *udhA*, product yield is lowest and increases by 55% to 1.25 mole product/mole glucose with *udhA* expression. A maximum yield of 1.44 mole product/mole glucose consumed is achieved with *udhA* overexpression in a *pntAB* knockout background, a 15% and 79% increase over the *udhA*-expressing and *udhA*-deletion strains. These results indicate that the presence of *udhA* has a positive effect on product yield. Therefore, it appears that NADPH availability is *udhA* limiting under aerobic conditions.

Table 3-6. Production of (S)-2 chloropropionate in *E. coli* shake flask experiments under aerobic conditions in *pntAB*-knockout strains with varying degrees of *udhA* expression in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression, (-) denotes transhydrogenase deletion, and (++) denotes transhydrogenase overexpression. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MBS603 pCAA43 pDHC29 *pntAB- udhA-*	0.8075 ± 0.0408	42.71 ± 1.113
MBS602 pCAA43 pDHC29 *pntAB- udhA+*	1.252 ± 0.0669	11.59 ± 0.7369
MBS602 pCAA43 *udhA* *pntAB- udhA++*	1.442 ± 0.0684	7.189 ± 0.3529

Complementation strains using single deletion background strains such as MBS601 and MBS602 presented a challenge due to issues with plasmid stability and cell growth. Complementation strains using a double deletion background (MBS603) coupled with either *udhA* or *pntAB* expression plasmids were more viable. However, results obtained with the two constructed complementation strains (MBS603 pCAA43 *udhA*, MBS603 pCAA43 *pntAB*) fell short of those obtained with single deletion strains, due to a lack of knowledge on factors such as gene dosage, gene expression, etc (Table 3-6).

Table 3-7. Production of (S)-2 chloropropionate in *E. coli* shake flask experiments under aerobic conditions in complementation strains in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression and (-) denotes transhydrogenase deletion. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MBS603 pCAA43 *udhA* *udhA+ pntAB-*	0.9252 ± 0.0057	39.41 ± 0.5493
MBS602 pCA43 pDHC29 *udhA+ pntAB-*	1.252 ± 0.0669	11.59 ± 0.7369
MBS603 pCAA43 *pntAB* *udhA- pntAB+*	0.2151 ± 0.0023	40.87 ± 0.3646
MBS601 pCAA43 pDHC29 *udhA- pntAB+*	0.7271 ± 0.0079	42.23 ± 0.8095

The results of the (S)-2 chloroproprionate system evaluated under aerobic conditions at 37°C and 250 rpm in defined M9 medium showed that the presence of *udhA* in mutant E. coli strains expressing the 2-haloacrylate reductase gene catalyzing the NADPH-dependent reduction of 2-chloroacrylate to (S)-2 chloropropionate increases NADPH availability that can, in turn, be used to increase the yield of NADPH-dependent products. Product yield is greatest in a single *pntAB* deletion strain with *udhA* overexpression, resulting in a 150% increase in NADPH-dependent production of (S)-2 chloropropionate over the wild-type control strain (MG1655 pCAA43 pDHC29) from 0.571 mole product/mole glucose to 1.44 mole product/mole glucose. Additionally, when *udhA* is expressed in a clean background lacking both inherent *pntAB* and *udhA*

(MBS603 pCAA43 *udhA*), product yield is 62% greater than the wild-type control strain and 6.9% greater than *udhA* overexpression strain in a wild-type background (MG1655 pCAA43 *udhA*). Similar to findings by Lee et al. (2010) and Sanchez et al. (2006), these results suggest that *udhA* overexpression has a positive effect on product per glucose yield.

Contrastingly, mutant strains overexpressing *pntAB* had the lowest observed product yields. Product yield suffered most in a *pntAB-udhA* mutant strain overexpressing *pntAB* (MBS603 pCAA43 *pntAB*) and was 62% lower than the wild-type control strain (0.215 mole product/mole glucose vs. 0.571 mole product/mole glucose). A slight drop in product yield occurred when *pntAB* was overexpressed in a wild-type background, decreasing by 9.1% compared to the control (0.519 mole product/mole glucose vs. 0.571 mole product/mole glucose). These findings suggest that *pntAB* erexpression, particularly in the absence of *udhA*, has a detrimental effect on NADPH-dependent product yield and NADPH availability in the presence of an electron sink.

When inherent *pntAB* expression is accompanied by *udhA* deletion (MBS601) in aerobic conditions, a higher product yield is still achieved, though at a fraction of the amount when *udhA* expression is accompanied by *pntAB* deletion (MBS602) (Table 3-4 and Table 3-5), suggesting that *udhA* expression has a more prominent role in NADPH generation in the presence of an electron sink. Interestingly, a double deletion strain containing neither of the two transhydrogenase isoforms produced 42% more product than the control strain, indicating a greater NADPH pool in the absence of both *pntAB* and *udhA*.

In summary, aerobic (S)-2 chloropropionate production experiments suggest that, in the presence of an electron sink, the soluble, energy-independent transhydrogenase *udhA* has a greater effect on driving NADPH regeneration in whole cell biocatalysis. Furthermore, *pntAB* overexpression reduces the NADPH pool.

3.4.2 Anaerobic (S)-2-chloropropionate production experiment results

Anaerobic shake tube experiments were performed with mutant and control strains in defined M9 medium utilizing glucose as the carbon source. Mutant *E. coli* strain cell growth was comparable to the control, indicating limited growth impairment with the exception of the *pntAB* deletion strain (MBS602 pCAA43 pDHC29) (Fig. 3-6).

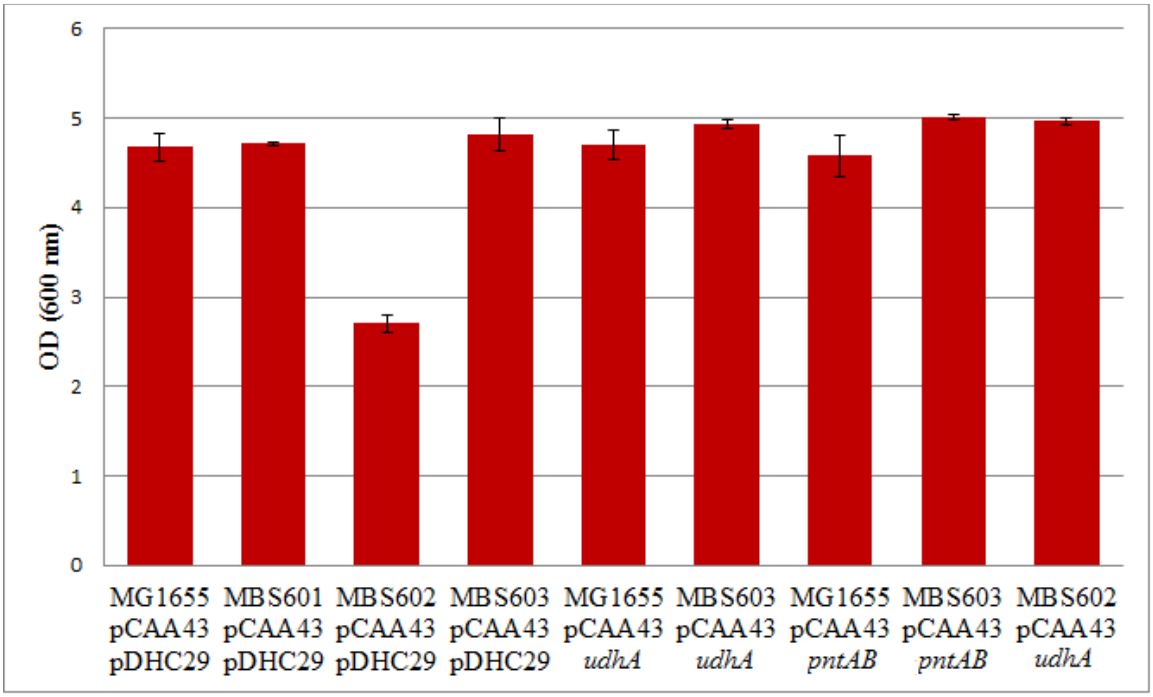

Figure 3-6. Cell growth of control (MG1655 pCAA43 pDHC29) and modified *E. coli* strains (MBS601, Δ*udhA*; MBS602, Δ*pntAB*; MBS603, Δ*pntAB*Δ*udhA*) under aerobic conditions in LB medium with IPTG induction for 18 hours at 30°C and 250 rpm, subcultured into the anaerobic shake tubes.

When evaluating the three mutant background strains against the control in an anaerobic environment, a trend similar to the aerobic (S)-2 chloropropionate production experiments was observed. However, only two of the three mutant strains exhibited higher product yield than the control, with the single *udhA* knockout strain being the exception (Table 3-8). The single *pntAB* knockout strain (MBS602) had the highest yield, followed by the double deletion (MBS603) and single *udhA* knockout strain (MBS601). MBS602 produced 1.9 times (S)-2 chloropropionate than the control with 90.9% more NADPH. MBS603 produced 1.4 times (S)-2 chloropropionate and 38% more NADPH than the control. In contrast to the aerobic (S)-2 chloropropionate production experiments, the *udhA* knockout strain (MBS601) did have a higher product yield in comparison to the control. Rather, product yield dropped by 20%, from 0.552 mole product/mole glucose to 0.440 mole product/mole glucose.

Table 3-8. Production of (S)-2 chloropropionate in anaerobic shake tubes by *E. coli* strains with wild-type and mutant backgrounds in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression and (-) denotes transhydrogenase deletion. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MG1655 pCAA43 pDHC29 *udhA+ pntAB+*	0.552 ± 0.0164	31.87 ± 0.6574
MBS601 pCAA43 pDHC29 *udhA- pntAB+*	0.440 ± 0.0006	29.54 ± 0.0873
MBS602 pCAA43 pDHC29 *udhA+ pntAB-*	1.054 ± 0.0519	8.337 ± 0.7994
MBS603 pCAA43 pDHC29 *udhA- pntAB-*	0.763 ± 0.0260	15.52 ± 1.035

Similar to the aerobic (S)-2 chloropropionate production results, increasing levels of *pntAB* decrease product yield in *udhA*-expressing strains (Table 3-9). The *pntAB* overexpression (*pntAB*++) strain has the lowest product yield. Both product yield and NADPH levels increase by 52% in the *udhA*+ *pntAB*+ strain (MG1655 pCAA43 pDHC29) in comparison to the *pntAB* overexpression strain. Furthermore, when *pntAB* is removed from the strain altogether (*pntAB*-), both product yield and NADPH levels are maximized in this set of strains to 1.05 mole product/mole glucose. This *pntAB* deletion strain improves both product yield and the NADPH pool by 190% and 91% over the *pntAB* overexpression and the *pntAB* expression strains, respectively. The presence of

pntAB evidently decreases product yield, with decreasing product yield observed with increasing *pntAB* expression levels.

Table 3-9. Production of (S)-2 chloropropionate in *E. coli* shake flask experiments under anaerobic conditions in *udhA*-expressing strains with varying degrees of *pntAB* expression in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression, (-) denotes transhydrogenase deletion, and (++) denotes transhydrogenase overexpression. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MG1655 pCAA43 *pntAB* *udhA*+ *pntAB*++	0.3626 ± 0.0004	28.66 ± 0.1711
MG1655 pCAA43 pDHC29 *udhA*+ *pntAB*+	0.5522 ± 0.0164	31.87 ± 0.6574
MBS602 pCAA43 pDHC29 *udhA*+ *pntAB*-	1.054 ± 0.0519	8.337 ± 0.7994

When examining the effect of *pntAB* expression in the absence of *udhA*, a similar though less pronounced trend is observed (Table 3-10). Product yield and NADPH levels are boosted 73% upon *pntAB* deletion, increasing from 0.440 mole product/mole glucose to 0.763 mole product/mole glucose. These findings support the previous conclusion that *pntAB* overexpression has a detrimental effect on NADPH-dependent product yield and NADPH availability in the presence of an electron sink and is more pronounced in the absence of *udhA* expression. However, when comparing the effects of *pntAB* deletion in both a *udhA*-deletion (MBS603 pCAA43 pDHC29) and *udhA*-expression strain (MBS602 pCAA43 pDHC29), we note that a greater product yield is achieved in the *udhA*-expressing strain (1.05 vs. 0.763 mole product/mole glucose), suggesting that the

presence of *udhA* may be beneficial in supplying NADPH for the production of (S)-2-chloropropionate.

Table 3-10. Production of (S)-2 chloropropionate in *E. coli* shake flask experiments under anaerobic conditions in *udhA*-knockout strains with varying degrees of *pntAB* expression in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression and (-) denotes transhydrogenase deletion. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MBS601 pCAA43 pDHC29 *udhA- pntAB+*	0.4402 ± 0.0006	29.54 ± 0.0873
MBS603 pCAA43 pDHC29 *udhA- pntAB-*	0.7628 ± 0.0260	15.52 ± 1.035

The effects of varying levels of *udhA* expression are examined more closely in the following set of strains with *pntAB* deletion (Table 3-11). In a double mutant background lacking both *pntAB* and *udhA*, product yield is lowest. In contrast to results obtained under aerobic conditions, *udhA* overexpression does not produce the maximal product yield. Instead, a *pntAB* deletion background strain with *udhA* expression (MBS602 pCAA43 pDHC29) has the greatest product yield (1.054 mole product/mole glucose) , only slightly above that of the *udhA* overexpression strain (MBS602 pCAA43 *udhA*). In fact, there is no significant difference in product yield of these two strains within error ($P > 0.05$, see Appendix). . These results deviate from those obtained under aerobic cultivation conditions. While they still suggest that the presence of *udhA* has a positive effect on product yield, it appears that (S)-2chloroproprionate production is not *udhA* limiting. Rather, NADPH availability is NADH limiting due to the fact that anaerobic

cellular metabolism cannot produce NADH via the TCA cycle as observed under aerobic conditions.

Table 3-11. Production of (S)-2 chloropropionate in *E. coli* shake flask experiments under anaerobic conditions in *pntAB*-knockout strains with varying degrees of *udhA* expression in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression, (-) denotes transhydrogenase deletion, and (++) denotes transhydrogenase overexpression. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MBS603 pCAA43 pDHC29 *pntAB- udhA-*	0.763 ± 0.0260	15.52 ± 1.035
MBS602 pCAA43 *udhA* *pntAB- udhA++*	1.016 ± 0.0441	6.116 ± 0.9355
MBS602 pCAA43 pDHC29 *pntAB- udhA+*	1.054 ± 0.0519	8.337 ± 0.7994

Complementation strains using single deletion background strains such as MBS601 and MBS602 presented a challenge due to issues with plasmid stability and cell growth. Complementation strains using a double deletion background (MBS603) coupled with either *udhA* or *pntAB* expression plasmids were more viable. However, results obtained with the two constructed complementation strains (MBS603 pCAA43 *udhA*, MBS603 pCAA43 *pntAB*) fell short of those obtained with single deletion strains, due to a lack of knowledge on factors such as gene dosage, gene expression, etc (Table 3-12).

Table 3-12. Production of (S)-2 chloropropionate in *E. coli* shake flask experiments under anaerobic conditions in complementation strains in defined M9 medium using glucose as the energy source where (+) denotes transhydrogenase expression and (-) denotes transhydrogenase deletion. Data shown are averages of triplicate experiments.

Strain	**Yield (mole product/mole glucose)**	**Glucose Consumption**
MBS603 pCAA43 *udhA* *udhA+ pntAB-*	0.724 ± 0.0316	24.30 ± 0.9975
MBS602 pCA43 pDHC29 *udhA+ pntAB-*	1.054 ± 0.0519	8.337 ± 0.7994
MBS603 pCAA43 *pntAB* *udhA- pntAB+*	0.150 ± 0.0062	27.78 ± 0.7222
MBS601 pCAA43 pDHC29 *udhA- pntAB+*	0.440 ± 0.0006	29.54 ± 0.0873

The metabolic role of the two transhydrogenase isoforms *pntAB* and *udhA* is still a matter of debate and is largely dependent upon the experimental conditions used (Chin 2009, Hua 2003, Lee 2010, Sanchez 2006, Sauer 2004). The mutant strains used in both the aerobic and anaerobic (S)-2 chloropropionate experiments in this study followed highly similar trends and suggest that, in the presence of an electron sink, the soluble, energy-independent transhydrogenase *udhA* has a greater effect on driving NADPH regeneration in whole cell biocatalysis and that *pntAB* overexpression reduces the NADPH pool.

Overexpressing *udhA* increased product yield per glucose significantly while *pntAB* overexpression had the opposite effect (Fig. 3-7). Thus, similar to a study by Chin

et al. (2009), it appears that *pntAB* does not readily transfer reducing equivalents from NADH to NADPH in the given system. This finding is in contrast to a study performed by Weckbecker and Hummel (2004), wherein *pntAB* overexpression increased (R)-phenylethanol production from 19% to 66%, suggesting sufficient NADPH regeneration via *pntAB*. Weckbecker and Hummel evaluated NADPH supply by coupling three enzymatic reactions incorporating the NAD+-dependent formate dehydrogenase, wherein formate acted as the source of reducing equivalents and could produce one (R)-phenylethanol per formate consumed. It appears that, given a one-step system with an electron sink, *pntAB* is not a major source of NADPH and is, in fact detrimental to NADPH regeneration as evidenced by the significant increase in (S)-2chloropropionate yield per glucose in both aerobic and anaerobic conditions over the control. (S)-2 chloropropionate yields with *udhA* overexpression increased significantly, supporting reported results by Lee et al. (2010) and Sanchez et al. (2006) and suggesting increased NADPH availability due to *udhA* overexpression.

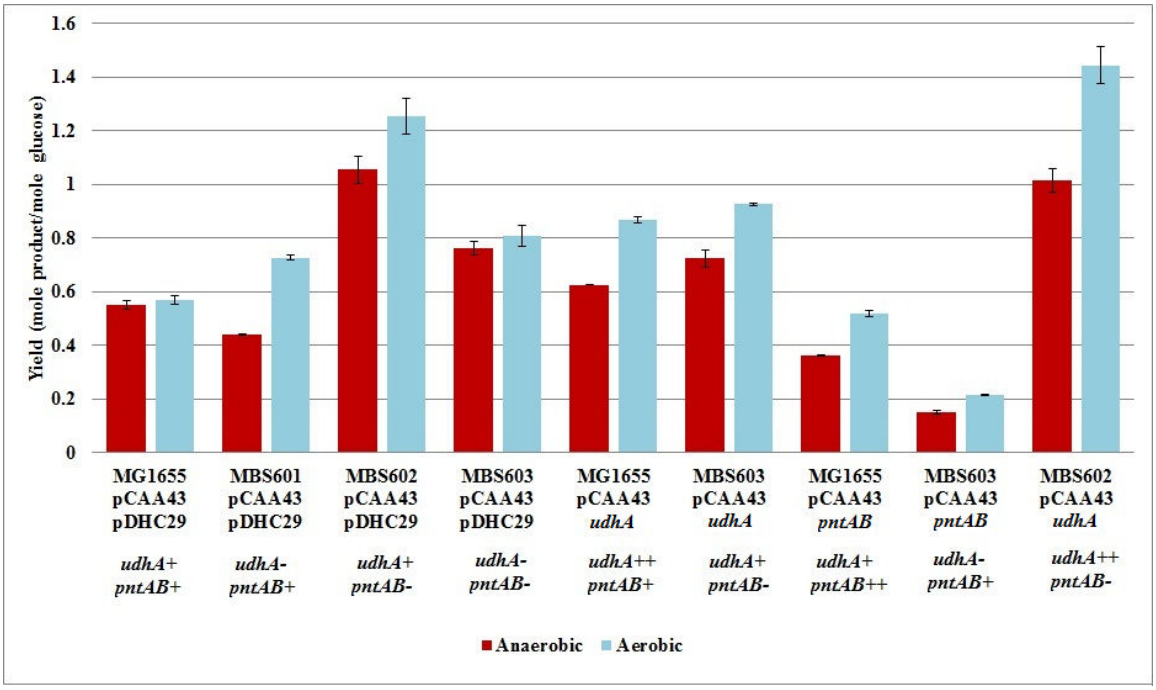

Figure 3-7. Aerobic and anaerobic product yield per glucose consumption at 6 hours for seven mutant *E. coli* strains and a control (MG1655 pCAA43 pDHC29) where (+) denotes transhydrogenase expression, (-) denotes transhydrogenase deletion, and (++) denotes transhydrogenase overexpression.

Chapter 4

4. Effects of Pyridine Nucleotide Transhydrogenases on the Production of Poly (3-hydroxybutyrate) in *Escherichia coli*

4.1 Abstract

The effects of the energy-linked, membrane-bound (*pntAB*) and soluble, energy-independent (*udhA*) transhydrogenase are examined in a poly(3-hyroxybutyrate) production system that utilizes acetoacetyl-CoA reductase to convert acetyl-CoA to PHB. The second step in the synthesis of PHB requires NADPH as a cofactor. Mutant deletion strains lacking either one or both of the pyridine nucleotide transhydrogenases were shown to increase NADPH availability, resulting in increased concentration and yield of PHB in recombinant *Escherichia coli*. Expression of the phb operon from *Alcaligenes eutrophus* H16 resulted in increased PHB concentration, most notably in the double deletion mutant strain (MG1655Δ*pntAB*Δ*udhA* pAet29) from 2.11 g/L to 5.07 g/L, a nearly 2.5 fold increase relative to the control. PHB yield was increased from 61% to 90% PHB per g of total dry cell weight as well. These results provide insight on the roles of pyridine nucleotide transhydrogenases anddemonstrate the effectiveness of cofactor manipulation in metabolic engineering.

4.2 Introduction

As mentioned in the previous chapter, cofactor pairs like NADH/NADPH are essential in the syntheses of industrially useful compounds (Gottschalk 1986). These

cofactor pairs are utilized in redox reactions and act by transforming in a reversible manner between oxidized and reduced states. As the production of high-value compounds like antibiotics, biodegradeable polymers, and chiral alcohols, to name a few, are dependent upon the availability of such low molecular weight cofactors, they act as a useful control parameter when studying cellular processes in the attempt to achieve specific metabolic engineering goals. Unfortunately, their high cost hinders their use in large-scale industrial processes. Studies implementing whole-cell biocatalysis, wherein effective cofactor regeneration can be achieved, are used. These whole-cell systems present viable, cost-effective approaches for supplying and regenerating cofactor, but are limited by indigenous regeneration rates.

This work focuses on the manipulating and characterizing NADPH availability in mutant *Escherichia coli* strains through the application of rational genetic modifications. Availability is assessed by determining product yields of cofactor-dependent products in *E. coli*. This objective is achieved using classic metabolic engineering strategies such as the deletion of specific enzymes, using whole-cell fermentation systems to allow for more robust and cost-effective compound synthesis. Specifically, NADPH availability is manipulated in an effort to increase product yield of the biodegradable polymer, poly (3-hydroxybutyrate) (PHB).

PHB is naturally produced by a variety of bacteria, accumulating as granules within the cytoplasm as an intracellular energy reserve (Anderson, 1990). The biopolymer is a biodegradable, semi-crystalline thermoplastic that possesses biocompatible properties (Doi, 1990). The PHB synthesis pathway and associated enzymes have been studied previously in *Alcaligenes eutrophus* by Haywood et al. (1988,

1989), and the phbC-phbA-phbB operon containing the three genes encoding the synthase-thiolase-reductase enzymes, respectively, have been cloned an analyzed as well (Steinbuchel, 1991). Previous studies have shown that, when cloned and expressed in *E. coli,* a large amount of PHB accumulation is observed (Kusaka 1997, Sanchez 2006, Schubert 1988, Slater 1988).

In this study, the effect of the two transhydrogenases, the membrane-bound, energy-linked isoform *pntAB* and the soluble, energy-independent isoform *udhA*, were assessed by constructing unique mutant deletion strains containing the PHB operon needed for PHB synthesis from acetyl-CoA in *E. coli* as indicated in the pathway depicted n Figure 4-1. The first enzyme when synthesizing PHB from acetyl-CoA, β-ketothiolase, is encoded by the gene phbA. Two acetyl-CoA molecules are transformed in a reversible condensation reaction into acetoacetyl-CoA, and this intermediate is reduced to 3-hydroxybutryl-CoA by the NADPH-linked acetoacetyl-CoA reductase (phbB). The polymerization of 3-hydroxybutryl-CoA is catalyzed by PHB synthase (phbC) to form the biodegradable polymer poly(3-hydroxybutryrate), or PHB. (Kusaka 2007, Sanchez 2006).

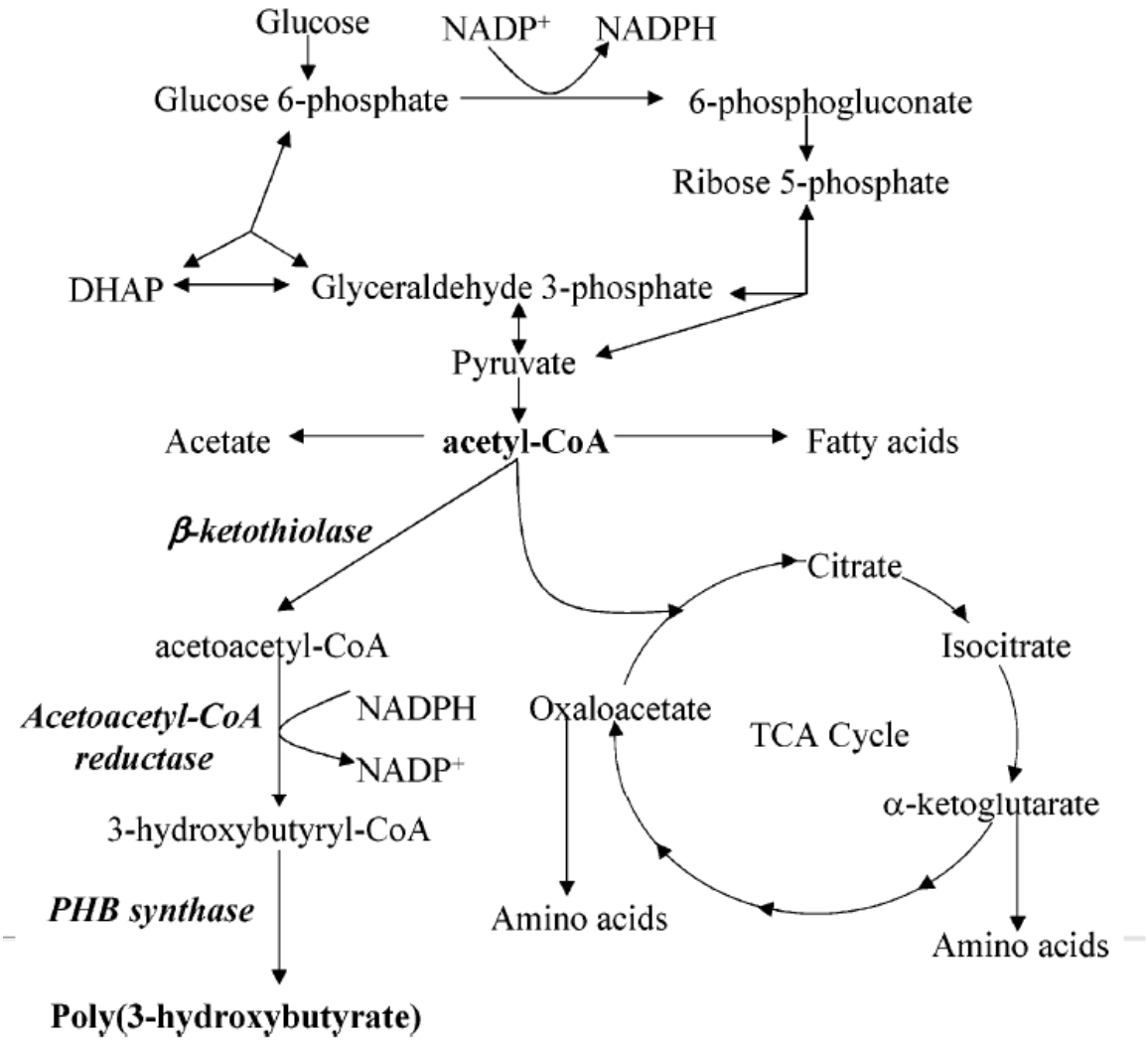

Figure 4-1. Synthesis pathway for poly(3-hydroxybutyrate) in *Escherichia coli* under aerobic conditions. (Sanchez 2006)

PHB is chosen as a model system when investigating the effects of NADPH availability because the second enzyme in the process of converting acetyl-CoA to PHB, acetoacetyl-CoA reductase, requires NADPH as a cofactor (Fig. 4-1). As a result, the activity of this enzyme coupled with the NADPH pool present themselves as important regulating factors for PHB synthesis (Anderson 1990, Srienc 1998, Sanchez 2006).

Additionally, the production of PHB yields such large quantities that it acts as an NADPH drain (Sanchez, 2006).

A previous study by Sanchez et al. demonstrated the effectiveness of cofactor manipulation as a tool in metabolic engineering. In the study, overexpression of the soluble transhydrogenase, *udhA*, alongside expression of the phb operon in *Escherichia coli* was reported to increase both the productivity and yield of PHB in vivo.

The two transhydrogenease isoforms in this study transfer electrons between NAD and NADP as shown in the following equation:

$$NADH + NADP^+ \leftrightarrow NAD^+ + NADPH.$$

Here, we wish to extend upon findings from Sanchez et al. in an effort to gain a better understanding regarding the physiological role of pyridine nucleotide transhydrogenases in *E. coli* and the direction of the reaction they catalyze in NADPH shuffling. Wild-type (MG1655) and mutant deletion *E. coli* strains were transformed with the pAet29 plasmid encoding the phb operon, and the functionality of this system was demonstrated in PHB production experiments. The following results suggest that PHB yield can be increased upon increased NADPH availability.

4.3 Materials and methods

Chemicals used in the following procedures were purchased from Sigma-Aldrich (St. Louis, MO), unless denoted otherwise.

4.3.1 Bacterial strains and plasmids

The strains and plasmids used in this study are described in Table 4-1, below. Wild type (MG1655), single *udhA* (MBS601) and *pntAB* (MBS602) transhydrogenase mutants, and a double transhydrogenase mutant (MBS603) were transformed with plasmids pAet29, containing the PHB operon containing the genes phbA, phbB, and phbC required for the synthesis of PHB from acetyl-CoA in *E. coli*, and either p*PNTAB* carrying the *pntAB* gene or pUDHAC carrying the *udhA* gene. pDHC29 was used as a control. Construction of these strains is described in detail in section 2.1.2 of the Materials and Methods chapter.

Table 4-1. List of *E. coli* strains and plasmids used in this study.

	Genotype	References
Strain		
MG1655	Wild type *E. coli* ($F^- \lambda^-$)	ATCC 47076
MBS601	MG1655 Δ*udhA*	This study
MBS602	MG1655 Δ*pntAB*	This study
MBS603	MG1655 Δ*pntAB* Δ*udhA*	This study
Plasmids		
pDHC29	Cloning vector, Cm^R	(Phillips et al. 2000)
pAeT29	Encodes β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHB synthase enzymes from *Alcaligenes eutrophus,* Amp^R	ATCC 75207
p*PNTAB*	Proton-translocating pyridine nucleotide transhydrogenase gene *(pntAB)* from *E. coli*, in pDHC29, Cm^R	This study
pUDHAC	Soluble pyridine nucleotide transhydrogenase gene (*udhA*) from *E. coli* in pDHC29, Cm^R	This study

4.3.2 Culture medium

Luria-Bertani (LB) broth medium contained 10 g/L tryptone (Amresco, Solon, OH), 5 g/L yeast extract (BD Biosciences, Franklin Lakes, NJ) and 10 g/L NaCl. The LB medium was autoclaved at 121°C for 20-35 minutes, depending upon the volume of liquid being autoclaved. After autoclaving, glucose, antibiotics, and other various compounds were added aseptically as need be in accordance with each experiment. A cocktail mixture of ampicillin, carbenicillin, and oxacillin was added at a concentration of 200 mg/L. The LB medium used was supplemented with 2% glucose as specified in experimental procedures. (Sanchez 2006)

4.3.3 Cultivation conditions

Overnight precultures were prepared by inoculating a single colony into 5 mL LB supplemented with appropriate antibiotics with no glucose content and incubated overnight at 37 °C and 250 rpm. Cultures were inoculated with 1% v/v of the overnight preculture (e.g. 0.5 mL overnight preculture into a 250 mL flask containing 50 mL LB medium). Aerobic shake flask experiments were performed using 250 mL shake flasks containing 50 mL of LB media. The LB media was supplemented with a cocktail mixture of ampicillin, carbenicillin, and oxacillin to a final concentration of 200 mg/L and 2% glucose. Flasks were capped with foam stoppers and grown in in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT) at 37 °C and 250 rpm. Samples of the culture were taken at 24 and 31 hours and analyzed for poly(3-hydroxybutyrate) and extracellular metabolite production.

4.3.4 Inoculum preparation and conditions for poly(3-hydroxybutyrate) production experiments

For each experiment, wild-type, single *udhA* and *pntAB* transhydrogenase mutants, and the double transhydrogenase mutant were freshly transformed with the pAet29 plasmid. A single colony was restreaked onto a plate containing 200 mg/L of a cocktail mixture of ampicillin, carbenicillin, and oxacillin. A single colony was transferred into 5 mL LB supplemented with 200 mg/L of a cocktail mixture of ampicillin, carbenicillin, and oxacillin, and grown overnight aerobically at 37°C and 250 rpm in an Excella E25 rotary shaker (New Brunswick Scientific, Enfield, CT). Cells were inoculated at 1% v/v into 250 mL shake flasks containing 50 mL of LB media supplemented with 200 mg/L of a cocktail mixture of ampicillin, carbenicillin, and oxacillin and supplemented with 2% glucose. Flasks were grown aerobically at 37°C and 250 rpm.

4.3.5 Analytical techniques

4.3.5.1 Cell density

Cell density (OD) was taken at 600nm in a DU 800 spectrophotometer (Beckman Coulter, Irving, TX). Culture samples were diluted, as appropriate, with LB medium to maintain readings in the linear range of the spectrophotometer (0.1-0.4 OD_{600}).

4.3.5.2 Analysis of extracellular metabolites

In order to quantify extracellular metabolites, 1 mL of the fermentation broth was sampled and centrifuged at 13,000 g for 3 minutes in a microcentrifuge. The supernatant

was filtered through a 0.2 μm PTFE syringe filter and stored frozen for HPLC analysis. A Shimadzu-10A HPLC System (Shimadzu Scientific Instruments, Columbia, MD) equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A), and a differential refractive index detector, RI, (Waters, Milford, MA) was used to detect extracellular metabolites such as glucose, succinate, lactate, and acetate, among others. A solution of 2.5 mM H_2SO_4 was used as the mobile phase and operated at a0.5 mL/min flow rate. The column was operated at 55°C.

4.3.5.3 PHB extraction

Samples for PHB extraction were withdrawn aseptically after 24 and 31 hours of cultivation. A 20 mL sample was withdrawn from the aerobic shake flasks at each timepoint, washed three times with 0.15 N NaCl and resuspended in 15 mL of 0.15 N NaCl. Resuspended cells were transferred to preweighed and predried glass tubes and dried to constant weight using dry nitrogen. A control tube containing 15 mL of 0.15 N NaCl was concurrently dried to constant weight and subtracted from the sample's measured dry cell weight. Samples of PHB-containing dry cell mass were digested in 2 mL of concentrated H_2SO_4 for 45 minutes at 100°C on a heating block. After cooling on ice, 10 mL of 0.014 N H_2SO_4 was added to the tubes and vortexed to ensure rapid mixing. Extracted samples were diluted once more with 0.014 N H_2SO_4 to a final dilution factor ranging from 12-72, as needed. (Sanchez, 2006)

4.3.5.4 Analysis of PHB production

PHB production was quantified using a Shimadzu-10A HPLC System (Shimadzu Scientific Instruments, Columbia, MD) equipped with a cation-exchange column (HPX-87H, BioRad Labs, Hercules, CA), a UV detector (Shimadzu SPD-10A), and a differential refractive index detector, RI, (Waters, Milford, MA). Approximately 1 mL of the diluted extract was centrifuged at 13,000 g for 3 minutes in a microcentrifuge. The supernatant was filtered through a 0.2 μm PTFE syringe filter and stored chilled for HPLC analysis. The mobile phase used was a 2.5 mM H_2SO_4 solution at a flow rate of 0.6 mL/min. The column was operated at 55°C.

The amount of PHB produced was calculated based upon a method developed by Karr et al. (Karr et al., 1983). The absorbance of crotonic acid was measured at 210 nm with a retention time of 25.2 minutes. The amount of crotonic acid derived from PHB was calculated using a known standard calibration curve of crotonic acid. PHB content was estimated based upon the amount of crotonic acid detected using the previously determined conversion rate of PHB to crotonic acid of 1.81, as described by Taguchi et al. (Taguchi et al., 2001).

4.4 Results and discussion

Aerobic shake flask experiments were performed with control and mutant deletion strains in LB medium supplemented with 2% glucose. PHB biopolymers accumulated in the presence of glucose. Three mutant deletion strains were evaluated against a control strain (MG1655), each containing the PHB operon containing β-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHB synthase enzymes from *Alcaligenes*

eutrophus (pAet29). MG1655, MBS601, MBS602, and MBS603 were all freshly transformed with pAet29, and the experiments were performed under aerobic conditions in LB medium supplemented with a cocktail mixture of ampicillin, carbenicillin, and oxacillin at a concentration of 200 mg/L and 2% glucose.

The results of these experiments at 31 hours are shown in Table 4-2. The deletion of transhyrdogenases under aerobic conditions significantly increased the optical density of the culture while decreasing the acetate expression. Glucose consumption was significantly higher in the mutant deletion strains compared to the control, increasing by 81% in the *udhA* knockout strain and 62% in the double deletion strain. As suggested in a previous study by Sanchez et al. (2006), the slight decrease in acetate yield could be due to increased carbon utilization in redox balancing pathways, wherein a higher production rate of NADPH is balanced out by redirecting flux from the NADPH-producing TCA cycle to the non-NADPH-producing PEP-glyoxylate cycle in *E. coli* as described by Fischer et al. (2003). The PEP-glyoxylate cycle is known to oxidize two PEP molecules by means of acetyl-CoA, citrate, glyoxylate, and oxaloacetate to CO_2, regenerating one PEP molecule in the process. Fischer and Sauer reported an active PEP-glyoxylate cycle in glucose excess batch cultures of NADPH-overproducing phosphorglucose isomerase (*pgi*) mutant *E. coli* strains, suggesting its role in redox-cofactor balancing in a manner similar to that of *udhA*. *UdhA* has been reported to have a prominent role in NADPH abundant conditions, facilitating the reduction of NAD+ to NADH using NADPH as the electron donor (Canonaco 2001, Sauer 2004). Likewise, the PEP-glyoxylate cycle has potential role under conditions where NADPH formation rates exceed its consumption (Fischer, 2003).

The two mutant deletion strains, MBS601 pAet29 and MBS603 pAet29, consumed a considerably higher amount of glucose ($P < 0.05$, see Appendix). The mutant deletion strain MBS602 pAet29 did not produce reportable results due to suspected plasmid loss. Both strains produced significantly lower amounts of acetate ($P < 0.05$, see Appendix).

Table 4-2 Optical densities, glucose consumption, and specific metabolite yields of cultures of MG1655 control and mutant deletion strains under aerobic conditions in LB medium supplemented with 2% glucose as a carbon source at 31 hours. Standard deviations of triplicate cultures are shown in parentheses. ND = not detected due to plasmid loss.

Strain	**OD600 nm**	**Glucose Consumed (mM)**	**Acetate Concentration (mM)**
MG1655 pAet29 *udhA+ pntAB+*	11.33 ± 0.18	49.41 ± 2.17	54.07 ± 0.25
MBS601 pAet29 *udhA- pntAB+*	18.64 ± 0.87	89.62 ± 6.61	48.18 ± 1.08
MBS602 pAet29 *udhA+ pntAB-*	ND	ND	ND
MBS603 pAet29 *udhA- pntAB-*	22.06 ± 0.64	79.81 ± 2.45	47.76 ± 0.69

The reduced concentration of acetate in mutant deletion strains substantiates increased demand for acetyl-CoA. Acetate production decreases by 11% in the *udhA* deletion strain and by 12% in the double deletion strain (Δ*pntAB* Δ*udhA*).

Results on PHB concentration at 31 hours during the shake flask study are shown in Figure 4-2. PHB concentration was highest in the double deletion strain lacking both transhydrogenases (MBS603 pAet29), which produced 5.07 g/L of PHB during 31 hours of growth using glucose as the sole carbon source. The control strain produced 2.11 g/L of PHB. Therefore, the double deletion strain exhibited a 140% increase in PHB levels over the control. The single deletion strain (MBS601 pAet29) lacking *udhA* produced 4.46 g/L PHB after 31 hours of cultivation, which represents a 110% increase in PHB levels over the control. These results conform to those reported by Lee et al. (1996). Lee et al. concluded that one of the most critical regulating factors for PHB synthesis in recombinant *E. coli* was NADPH availability. Additionally, they reported that low citrate synthase activity, which catalyzes the funneling of acetyl-CoA into the TCA cycle, contributes to higher PHB synthesis, suggesting that both NADPH and acetyl-CoA availability are pertinent for the efficient synthesis of PHB in *E. coli*.

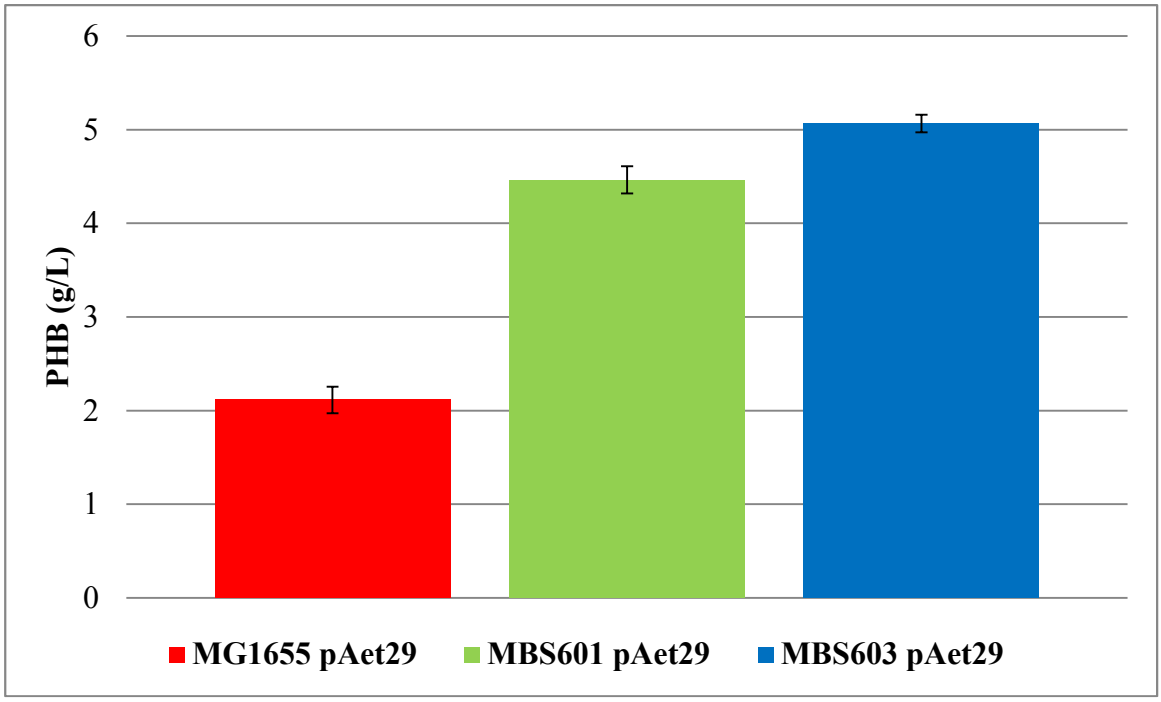

Figure 4-2 PHB concentration of control and mutant deletion strains after 31 hours of cultivation in aerobic shake flask experiments in LB medium supplemented with 2% glucose. MBS601 = MG1655Δ*udhA*, MBS603 = MG1655Δ*pntAB*Δ*udhA*. Error bars represent standard deviations of triplicate samples.

Figure 4-3 shows the PHB yield obtained during the shake flask study. PHB yield is reported as the percentage of PHB per gram total dry cell weight. Both mutant deletion strains increased PHB yield, though only slightly in the *udhA* deletion strain (from 61% to 65%). The double deletion strain, on the other hand, had a significantly higher PHB yield than the control ($P < 0.05$, see Appendix), increasing it from 61% to 90%. These results indicate that a larger proportion of the cells' resources is being directed towards PHB production rather than residual cell mass production, wherein residual cell mass is defined as total dry cell weight minus grams of PHB produced (Sanchez, 2006).

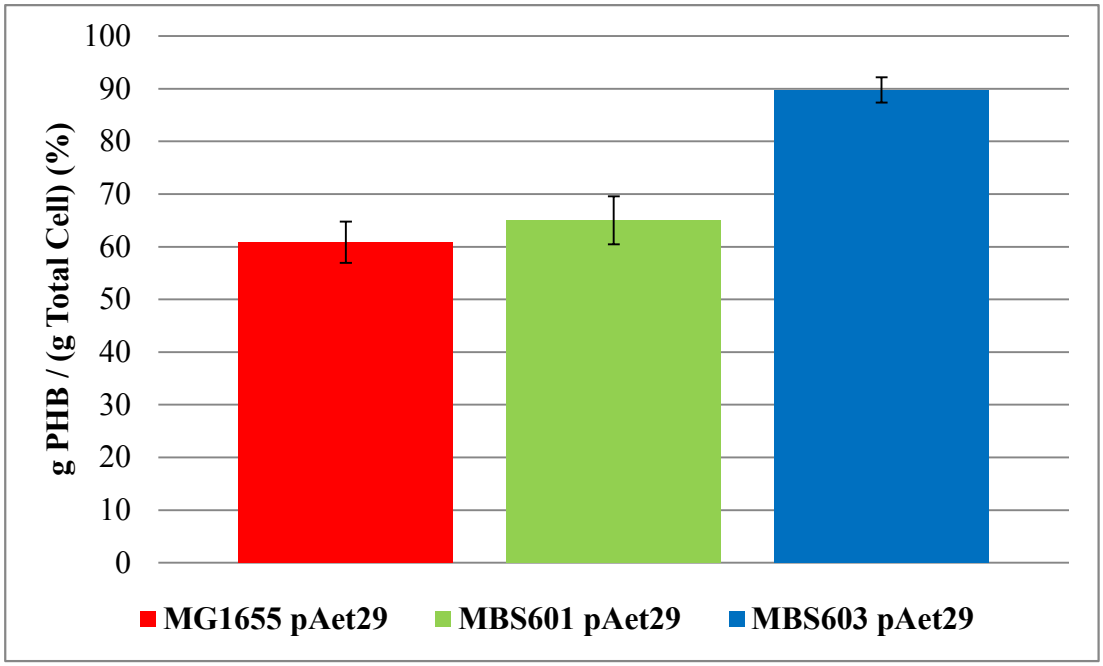

Figure 4-3 PHB yield of control and mutant deletion strains after 31 hours of cultivation in aerobic shake flask experiments in LB medium supplemented with 2% glucose. MBS601 = MG1655Δ*udhA*, MBS603 = MG1655Δ*pntAB*Δ*udhA*. Error bars represent standard deviations of triplicate samples.

This study aimed to determine transhydrogenase effects on NADPH availability, which was measured through the synthesis of PHB. The results suggest that increased NADPH availability, achieved through single and double mutant deletion strains, promote PHB synthesis. High levels and yields of PHB were obtained within 31 hours of culturing under aerobic conditions. Cofactor manipulation using transhydrogenases was shown to be an effective tool when attempting to increase productivity of cofactor-dependent compounds. Future work can be done to investigate the effects of transhydrogenase overexpression in control and mutant backgrounds in addition to the effects of a *pntAB* deletion strain.

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Appendix

Appendix A – Statistical analysis of aerobic (S)-2-chloropropionate production results

Aerobic (S)-2-chloroproprionate production experiments:
Product Yield/Glucose consumption

Groups	*Count*	*Sum*	*Average*	*Variance*
MG1655 pCAA43 pDHC29	3	1.71	0.570665	0.00026
MBS603 pCAA43 pDHC29	3	2.42	0.807538	0.00166
MBS602 pCAA43 pDHC29	3	3.76	1.25223	0.00448
MBS601 pCAA43 pDHC29	3	2.18	0.727108	6.2E-05
MG1655 pCAA43 *pntAB*+	3	1.56	0.519068	0.00012
MG1655 pCAA43 *udhA*+	3	2.6	0.865572	0.00013
MBS603 pCAA43 *pntAB*+	3	0.65	0.215074	5.2E-06
MBS603 pCAA43 *udhA*+	3	2.78	0.925205	3.3E-05

ANOVA Results

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.0101	7	0.28716	339.71	3.5E-16	2.6572
Within Groups	0.01352	16	0.00085			
Total	2.02362	23				

Aerobic (S)-2-chloroproprionate production experiments: Product Yield/Glucose consumption in *udhA*-expressing strains

Groups	*Count*	*Sum*	*Average*	*Variance*
MG1655 pCAA43 *pntAB*	3	1.5572	0.51907	0.000125
MG1655 pCAA43 pDHC29	3	1.712	0.57067	0.000265
MBS602 pCAA43 pDHC29	3	3.7567	1.25223	0.00448

ANOVA Results

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.00472	2	0.502	309.5452	8.844E-07	5.143253
Within Groups	0.00974	6	0.002			
Total	1.01446	8				

Aerobic (S)-2-chloroproprionate production experiments: Product Yield/Glucose consumption in *udhA*-deletion strains

Groups	*Count*	*Sum*	*Average*	*Variance*
MBS601 pCAA43 pDHC29	3	2.18	0.727	6.18E-05
MBS603 pCAA43 pDHC29	3	2.42	0.808	0.001663

ANOVA Results

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0097	1	0.01	11.25	0.02847	7.708647
Within Groups	0.00345	4	9E-04			
Total	0.01315	5				

Aerobic (S)-2-chloroproprionate production experiments: Product Yield/Glucose consumption in *pntAB* deletion strains

Groups	*Count*	*Sum*	*Average*	*Variance*
MBS603 pCAA43 pDHC29	3	2.42	0.808	0.002
MBS602 pCAA43 pDHC29	3	3.76	1.252	0.004
MBS602 pCAA43 *udhA*	3	4.33	1.442	0.005

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.63682	2	0.318	88.25	3.6E-05	5.143
Within Groups	0.02165	6	0.004			
Total	0.65847	8				

Appendix B – Statistical analysis of anaerobic (S)-2-chloropropionate production results

Anaerobic (S)-2-chloroproprionate production experiments: Product Yield/Glucose consumption

Groups	*Count*	*Sum*	*Average*	*Variance*
MG1655 pCAA43 pDHC29	3	1.656741	0.552247	0.00027
MBS603 pCAA43 pDHC29	3	2.288473	0.762824	0.000677
MBS602 pCAA43 pDHC29	3	3.161219	1.05374	0.002693
MBS601 pCAA43 pDHC29	3	1.320505	0.440168	3.39E-07
MG1655 pCAA43 *pntAB*+	3	1.08773	0.362577	1.51E-07
MG1655 pCAA43 *udhA*+	3	1.877327	0.625776	8.84E-07
MBS603 pCAA43 *pntAB*+	3	0.449678	0.149893	3.78E-05
MBS603 pCAA43 *udhA*+	3	2.171618	0.723873	0.001

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.599349	7	0.2285	390.666	1.15E-16	2.657197
Within Groups	0.009357	16	0.0006			
Total	1.608707	23				

Anaerobic (S)-2-chloroproprionate production experiments: Product Yield/Glucose consumption in *udhA*-expressing strains

Groups	*Count*	*Sum*	*Average*	*Variance*
MG1655 pCAA43 *pntAB*	3	1.0877	0.362577	2E-07
MG1655 pCAA43 pDHC29	3	1.6567	0.552247	0.0003
MBS602 pCAA43 pDHC29	3	3.1612	1.05374	0.0027

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.76518	2	0.3826	387.35	4.54E-07	5.14325
Within Groups	0.00593	6	0.001			
Total	0.7711	8				

Anaerobic (S)-2-chloroproprionate production experiments: Product Yield/Glucose consumption in *udhA*-deletion strains

Groups	*Count*	*Sum*	*Average*	*Variance*
MBS601 pCAA43 pDHC29	3	1.3205	0.4402	3E-07
MBS603 pCAA43 pDHC29	3	2.2885	0.7628	0.0007

ANOVA Results

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.15616	1	0.1562	461.35	2.78E-05	7.70865
Within Groups	0.00135	4	0.0003			
Total	0.15751	5				

Anaerobic (S)-2-chloroproprionate production experiments: Product Yield/Glucose consumption in *pntAB*-deletion strains

Groups	*Count*	*Sum*	*Average*	*Variance*
MBS603 pCAA43 pDHC29	3	2.2885	0.7628	0.0007
MBS602 pCAA43 pDHC29	3	3.1612	1.0537	0.0027
MBS602 pCAA43 *udhA*	3	3.0489	1.0163	0.0019

ANOVA Results

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.15029	2	0.0751	42.436	0.0003	5.14325
Within Groups	0.01062	6	0.0018			
Total	0.16091	8				

Anaerobic (S)-2-chloroproprionate production experiments: Product Yield/Glucose consumption in *pntAB*-deletion strains, *udhA*-expressing strains

Groups	*Count*	*Sum*	*Average*	*Variance*
MBS602 pCAA43 pDHC29	3	3.16	1.05374	0.0027
MBS602 pCAA43 *udhA*	3	3.05	1.01631	0.0019

ANOVA Results

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.0021	1	0.0021	0.9066	0.39494	7.70865
Within Groups	0.00927	4	0.00232			
Total	0.01137	5				

Appendix C – Statistical analysis of aerobic poly(3-hydroxybutyrate) production results

Glucose consumption (mM) ttest at 31 hours:

Strain A	**Strain B**	**P-Value**
MG1655 pAet29	MBS601 pAet29	5.58 E-4
MG1655 pAet29	MBS603 pAet29	9.01 E-05

Acetate concentration (mM) ttest at 31 hours:

Strain A	**Strain B**	**P-Value**
MG1655 pAet29	MBS601 pAet29	7.63 E-4
MG1655 pAet29	MBS603 pAet29	1.18 E-05

PHB yield per g total (%) ttest at 31 hours:

Strain A	**Strain B**	**P-Value**
MG1655 pAet29	MBS601 pAet29	0.297
MG1655 pAet29	MBS603 pAet29	3.96 E-4

PHB concentration (g/L) ttest at 31 hours:

Strain A	**Strain B**	**P-Value**
MG1655 pAet29	MBS601 pAet29	3.61 E-5
MG1655 pAet29	MBS603 pAet29	7.34 E-6